PCT/EP 03/08701

10/522883

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Patentanmeldung Nr.	Patent application No.	Demande de brevet n°

02291996.3

PRIORITY
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Anmeldung Nr.:
Application no.: **02291996.3**
Demande n°:

Anmeldetag:
Date of filing: **08/08/02**
Date de dépôt:

Anmelder:
Applicant(s):
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Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:
IL-7 drug substance, IL-7 comprising composition, preparation and uses thereof

In Anspruch genommene Prioriät(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat: State: Pays:	Tag: Date: Date:	Aktenzeichen: File no. Numéro de dépôt:

Internationale Patentklassifikation:
International Patent classification:
Classification internationale des brevets:
C12N15/24, C07K14/54, A61K38/20, C07K16/24, C12N15/62

Am Anmeldetag benannte Vertragstaaten:
Contracting states designated at date of filing: **AT/BG/BE/CH/CY/CZ/DE/DK/EE/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/**
Etats contractants désignés lors du depôt:

Bemerkungen:
Remarks:
Remarques:

The applicant's name at the time of filling of the application was as
follows: BIOTECH INFLECTION POINT SA BIP, Vanves, France.
The registration of the changes has taken effect on 11.06.03.
See for the original title of the application, page 1 of the description.

L-7 DRUG SUBSTANCE, COMPOSITION, PREPARATION AND USES

The present invention relates, generally, to the fields of immunology and molecular biology. The invention discloses, more particularly, new and improved interleukin-7

5   drug substances, corresponding specific immunoreactive antibodies, as well as compositions comprising the same, their preparation and uses. The invention also discloses methods to characterize the impurity profile of a r-hIL-7 drug substance used for therapeutic purpose, as well as optimized nucleotide sequences encoding mammalian IL-7, recombinant expression vectors and methods for preparing and

10   purifying said polypeptides.

BACKGROUND OF THE INVENTION

B and T lymphocytes are the primary effector cells of the immune responses. Both

15   cell classes are considered to derive ultimately from hematopoietic stem cells in the mammalian bone marrow, via progenitor or precursor cells representing distinguishable stages in the differentiation of each class. Mature T cells develop principally in the thymus, presumably from a precursor cell which migrates from the bone marrow to the thymus at an early stage of T lymphocyte development.

20   Numbers of factors are active on mature peripheral B and T cells, including IL-1, IL-2, IL-4, IL-5, interferon gamma, BSF-2, neuroleukin, transforming growth factor beta and IL-7 (EP0314415).

"Interleukin-7" or "IL-7" refers to a mammalian endogenous secretory glycoprotein

25   which is capable of inducing proliferation of bone marrow-derived lymphocyte progenitors and precursors, including the specialized precursors known as pre-B cells. Originally derived from the stromal element of a bone marrow cell line, IL-7 is also secreted by thymic cells, intestinal epithelial cells, keratinocytes and generally all lymphoid tissues. EP0314415 describes mammalian interleukin-7 proteins and

DNAs encoding said proteins. The mature human IL-7 (r-hIL-7), exclusive of glycosylation, produced in *Escherichia coli*, which is described in EP0314415, exhibits a 17,387 Daltons molecular weight and displays a high activity *in vitro* on specific bioassays based on the proliferation of various lymphocytes populations.

5 Alternative designations for this molecule are "pre-B cell growth factor" and "lymphopoietin-1". Indeed, originally, IL-7 was disclosed as a cytokine whose principal activity was induction of precursor B cell proliferation (Namen A.E. et al.; Journal of Experimental medicine; 1988; 167:988-1002). IL-7 has more recently been disclosed as being involved in the survival and proliferation of thymocytes (T-

10 Cells) during early stage of T-cell development (Schluns K.S. et al.; Nature Immunology; 2000; 1(5):426-432). Fry and collaborators further identified IL-7 as a potent modulator of thymic-independent T-cell regeneration in a multifactorial action (Fry T.J. et al.; Blood; 2001; 97(6):1525-1533).

15 IL-7 has thus a great potential therapeutic use in the stimulation of the proliferation of T cell precursors, of antibody-secreting B cells, in the stimulation of antigen driven T-Cell peripheral expansion, and in the production of naïve T-Cells as well as other hematopoietic cell types. A particularly interesting therapeutic use of active IL-7 molecules is for immune reconstitution of lymphopenic patients: patients treated

20 for a cancer, patients having received a bone marrow or a Stem Cell transfer, patients presenting an acquired or genetic immune deficiency, elderly patients or any patients having low CD4 count. Other interests reside in the ability of IL-7 to produce new naïve CD4 T-Cells or to expand specific pools in order to produce or increase specific immune responses (Vaccine enhancement).

25

In view of its therapeutic potential, there is considerable interest in developing technologies for producing biologically active IL-7 polypeptides and corresponding drug substances. There is much interest in producing IL-7 drug substances or

pharmaceutical compositions that comply with the requirements of regulatory authorities and are suitable for efficient therapeutic uses.

A drug substance or pharmaceutical composition aimed at stimulating a global or specific immune reconstitution should be efficient on a long term, which implies that it should not trigger a specific immune reaction against its own active principle.

The present invention now shows, unexpectedly, that the long term activity of recombinant human IL-7 is mostly expressed by a specific 1-4; 2-5; 3-6 conformer. The present invention further shows that efficient drug substances should not only contain the above conformer as the major constituent, but should also be essentially devoid of other conformers or IL-7 molecular variants, previously considered as active products.

Up to the present invention, although the existence of the Cys: 1-4; 2-5; 3-6 conformer was hypothesized through computational modeling based on hypothetical GM-CSF or IL-4 conformation analogy, only IL-7 conformer exhibiting disulfide bridges Cys: 1-6; 2-5; 3-4 had been characterized by tryptic digest and mass spectrometry. This form was thought to constitute the major form of IL-7. In sharp contrasts therewith, the invention now proposes new polypeptide products and pharmaceutical compositions which are essentially devoid of such conformer. The invention also discloses advantageous production and purification methods to produce such polypeptides and compositions, which exhibit a high biological activity with reduced risk of undesired response, such as anti-IL-7 immune responses.

The present invention thus discloses improved methods for the preparation and uses of said purified conformer of human IL-7, devoid of the above mentioned product related substances or impurities, which exhibits a particularly advantageous long term in vivo activity, able to favor global or specific immune responses.

SUMMARY OF THE INVENTION

A first object of the invention relates to a purified IL-7 conformer, said IL-7
conformer comprising the following three disulfide bridges: Cys: 1-4 (Cys2- Cys92);
2-5 (Cys34- Cys129); 3-6 (Cys47- Cys141). As disclosed further below, the IL-7
conformer is preferably a human recombinant IL-7 conformer, and may be
glycosylated or unglycosylated.

An other object of this invention resides in a drug substance comprising an IL-7
conformer as described above and essentially devoid of product-related impurities
and product related substances, particularly of other conformers or dimers of IL-7.

The invention also relates to the use of a drug substance as described above in the
manufacture of a medicament ("drug product") or pharmaceutical composition.

The invention further relates to a pharmaceutical composition comprising an
effective amount of a drug substance as described above and one or more
pharmaceutically compatible carriers or excipients.

The invention also provides nucleic acid molecules coding for an IL-7 polypeptide.
The nucleic acid molecules comprise a sequence encoding an IL-7 polypeptide,
said sequence being optimized for the expression, in a recombinant host, of
biologically active IL-7, particularly of an IL-7 conformer according to the invention.
More specifically, the nucleic acid molecules provide for a limited expression of
truncated IL-7 polypeptides and increase the yields of production of biologically
active human IL-7 conformer. Particular sequences comprise a mutated or
inactivated Shine-Dalgarno like sequence (e.g., SEQ ID NO: 1). Other particular
sequences comprise a peptide signal causing efficient secretion of the polypeptide

(e.g., SEQ ID NO: 3). The invention also discloses and provides, for the first time, the nucleic acid sequence of the simian IL-7 (e.g., SEQ ID NO: 12).

The invention further provides vectors comprising a nucleic acid as mentioned above and recombinant host cells comprising said nucleic acid or said vector. The nucleic acids and vectors may be used to produce recombinant mammalian IL-7 polypeptides in various competent host cells, as well as for gene therapy purposes.

The invention also provides an antibody specifically immunoreactive with a human IL-7 conformer according to the invention, hybridoma cell lines that produce said monoclonal antibody, as well as compositions suitable for diagnosis, assay or therapy comprising said antibody.

A further aspect of this invention is a method of producing a (recombinant) IL-7 conformer as described above, from prokaryotic or eukaryotic host cells, as well as a method of detecting or measuring the presence of an IL-7 conformer in a sample or to characterize a sample.

In a particular aspect, the method of producing an IL-7 conformer or a drug substance as defined above comprises:

a)   providing a sample comprising IL-7 polypeptides, and

b)   purifying an IL-7 conformer as described above.

The invention also relates to a method of characterizing or qualifying a sample, comprising detecting or measuring, in said sample, the presence of an IL-7 conformer as described above. Such method is particularly suited and important for validation of clinical lots or batches, whereby samples comprising excessive IL-7 molecular variants and/or product related impurities are discarded or further treated.

Another object of the invention relates to the use of an IL-7 conformer obtained by a method as described above, for the manufacture of a pharmaceutical composition to cause or modulate an immune response in a subject, particularly to induce a prolonged lymphopoiesis stimulation and/or to amplify an immune response.

The invention also relates to the use of an IL-7 conformer obtained by a method as described above, for the manufacture of a pharmaceutical composition to prevent or treat a disease associated with an immunodeficiency.

The invention further relates to the use of an IL-7 conformer according to the invention as a tool for experimental and pharmacological use in monkeys.

LEGENDS TO THE FIGURES

Figure 1: Representation of the structure of *E Coli* expression vector ptac-hIL-7, which comprises SEQ ID NO:1.

Figure 2: Representation of the structure of *E Coli* expression vector ptac-sIL7opt, which comprises a sequence encoding simian IL-7 (corresponding to SEQ ID NO: 12 devoid of nucleotides no: 4 to 75, which encode the signal peptide).

Figure 3: Representation of the structure of mammalian (HEK-293, CHO or BHK) expression vector pcDNA-hPSIL-7 containing SEQ ID NO:3.

Figure 4: Aligned nucleotide sequences of human and monkey cDNA.

Figure 5: Aligned amino acid sequences of human and monkey protein.

Figure 6: Electrophoresis analysis of simian IL-7 PCR product.

Figure 7: Electrophoresis analysis of optimized simian IL-7 PCR product.

Figure 8: Electrophoresis analysis of human PS-IL-7 PCR product.

Figure 9: SDS-PAGE analysis of purified recombinant human deglycosylated IL-7 conformer: coomassie blue colored and silver stained.

Figure 10: Bioassay analysis of mammalian (human and simian) IL-7 proteins on pre-B cell line.

Figure 11: Peripheral blood CD4 T cell count of normal monkey treated with various recombinant IL-7.

Figure 12: Western Blot analysis of IL-7-treated monkey sera for the detection of anti-IL-7 antibodies.

Figure 13: Peripheral blood CD4 T cell count of irradiated monkey treated with various recombinant IL-7.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a purified IL-7 conformer, said IL-7 conformer comprising the following three disulfide bridges: Cys: 1-4 (Cys2- Cys92); 2-5 (Cys34- Cys129); 3-6 (Cys47- Cys141), to drug substances and pharmaceutical compositions comprising said IL-7 conformer as well as to methods for their production and therapeutic uses. As will be disclosed further below, the IL-7 conformer is preferably a human recombinant IL-7 conformer, and may be glycosylated or unglycosylated.

The invention is based on the unexpected discovery that the mid or long term *in vivo* biological activity of IL-7 is essentially attributable to a particular conformation of the molecule, not suspected so far, and that highly improved therapeutic efficiency is achieved by producing pharmaceutical compositions comprising said IL-7 conformer in a substantially pure form.

IL-7 Conformer

A first object of this invention resides in a purified or isolated IL-7 conformer, said IL-7 conformer comprising the following three disulfide bridges: Cys: 1-4 (Cys2-Cys92); 2-5 (Cys34- Cys129); 3-6 (Cys47- Cys141).

5   IL-7 primary sequence (human and simian) contains six highly conserved cysteine residues which are involved in the formation of three intramolecular disulfide bridges. As originally described by Goodwin, these disulfide bridges are necessary for bioactivity, beta-mercaptoethanol treatment of the IL-7 molecule fully abolishing its activity (Goodwin R.G. et al.; Proceedings of the National Academy of Sciences

10   of the USA; 1989; 86:302-306). From this point, according to the rank of the cysteine residues positions, various conformers are possible, among which : 1-6; 2-5; 3-4 or 1-4; 2-5; 3-6 or 1-4; 2-6; 3-5 or 1-5; 2-4; 3-6, plus various other conformers, including contiguous bridges.
Various teams used computational chemistry in order to suggest a conformational

15   model for the protein. From secondary structure prediction programs, Srinivasan and collaborators proposed the helical regions of IL-7 and, by analogy with the GM-CSF and IL-4 three dimensional structures, proposed a protein conformation which implied the following assignment for the three bridges: 1-4; 2-5; 3-6 (Srinivansan S. et al.; Protein Engineering (supplement); 1993; 6:107). Also, by homology modeling

20   studies, Kroemer and collaborators predicted a 3D model of hIL-7, based on homology to structures of human IL-2, IL-4, GM-CSF and GH serving as templates and connecting them together in an up-up-down-down topology. In the context of this common topology, the 3D model of human IL-7 favored the hypothesis of a unique disulfide pattern: the 1-4; 2-5; 3-6 disulfide design (Kroemer R.T. et al.;

25   Protein Engineering; 1996; 9(6):493-498). However, in 1997, from MALDI Mass spectrometry analysis of a human IL-7 produced by recombinant technology and from site directed mutagenesis exchanging serine for cysteine, Cosenza and colleagues established the assignment of the three disulfide bridges to the positions 1-6; 2-5; 3-4, in a way fully analogous to the intra-molecular disulfide bridges

experimentally determined for IL-4 (Cosenza L. et al.; Protein Science; 2000; 9:916-926 — Walter M.R. et al.; Journal of Biological Chemistry; 1992; 267:20371-20376). In this work, the mass spectrometry analysis of the peptide digest of the recombinant native molecule clearly demonstrated the reality of the Cys2-Cys141 (1-6) disulfide bridge and the site directed mutagenesis established the critical role for bioactivity of the Cys2-Cys141 (1-6) and Cys47-Cys92 (3-4) bridges. The two Cys/Ser IL-7 mutants allowing these two single bridges were able to show a good bioactivity (EC50 of 4X10-9 and 2X10-9 M) in the bioassay in comparison to EC50 of 2X10-10 for the three disulfide bridges molecule 1-6; 2-5; 3-4. Based on these experimental results, these three disulfide bridges 1-6; 2-5; 3-4 were assigned to the human Interleukin-7 and filed into the international protein data bank (Swiss Prot P13232, PDB code 1IL7).

The present invention now shows that the above three-dimensional structure, presented, demonstrated and accepted in the data bank until now, is incorrect and that the long term activity of recombinant human IL-7 is mostly expressed by a specific 1-4; 2-5; 3-6 conformer. The experimental results obtained differ from the previously reported disulfide bonds in human IL-7 (hIL-7) (Cosenza L. et al.; Journal of Biological Chemistry; 1997; 272:32995-33000) and show, for the first time, that long term active IL-7 compositions require the presence of a substantially pure particular IL-7 conformer which is in accordance with other models designed by homology modeling with GM-CSF and IL-4 conformations.

Within the context of the present invention, an "IL-7 polypeptide" designates a mammalian (e.g., human or simian) IL-7 polypeptide and, more preferably, a human IL-7 polypeptide, especially for uses as therapeutic or vaccine, or a simian IL-7 polypeptide, especially for use in non human primate experiments. Preferred IL-7 polypeptides of this invention comprise an amino acid sequence as described in EP 314 415 as well as any natural variants and homologs thereof. The sequence of

human IL-7 is available on genebanks. The typical wild-type protein comprises 152 amino acids and, optionally, an additional N-terminal methionine residue (SEQ ID Nos: 2 and 4). Variants thereof include, more preferably, natural allelic variants resulting from natural polymorphism, including SNPs, splicing variants, etc. In a

5 specific embodiment, the term IL-7 polypeptide is meant to designate a polypeptide having the sequence of SEQ ID NO: 2 or 4 or 13 [SEQ ID NO: 13 comprising or not the signal peptide (amino acids no: 2-25)], as well as natural variants thereof. The specific IL-7 polypeptide used in the present invention is preferably a recombinant IL-7, more preferably a recombinant human IL-7 conformer comprising the amino

10 acid sequence SEQ ID NO: 2 or 4.

A specific "conformer" of IL-7 designates an IL-7 polypeptide having a particular three-dimensional configuration.

15 The term "recombinant", as used herein, means that the polypeptide is obtained or derived from a recombinant expression system, i.e., from a culture of host cells (e.g., microbial or mammalian) engineered to contain a nucleic acid molecule encoding an IL-7 polypeptide. "Microbial" refers to recombinant proteins made in bacterial expression systems.

20

For medical use (including therapeutic, prophylactic, etc), the IL-7 conformer is preferably a recombinant human IL-7 conformer. In this regard, in a particular embodiment, the specific IL-7 conformer has the amino acid sequence of SEQ ID NO:4 and comprises the disulfide bridges Cys2-Cys92; Cys34-Cys129 and Cys47-

25. Cys141. In an other specific embodiment, the specific IL-7 conformer has the amino acid sequence of SEQ ID NO:4 and comprises an additional Methionine at the N-terminal position (i.e., SEQ ID NO:2).

For further uses, including experimental uses, the IL-7 conformer may be a recombinant simian IL-7 conformer. The recombinant simian IL-7 conformer preferably comprises amino acid sequence SEQ ID NO: 13 [SEQ ID NO: 13 comprising or not the signal peptide (amino acids no: 2-25)].

The IL-7 conformer of this invention may be glycosylated or unglycosylated. Many secreted proteins acquire covalently attached carbohydrate units posttranslationally. Glycosylation is frequently in the form of oligosaccharide units linked to proteins through an asparagine residue (situated in the consensus sequence: -Asn-X-Ser/Thr- where X is any amino acid except proline) or through serine/threonine amino acid side chains, giving N-glycosidic or O-glycosidic bonds, respectively. Both the structure and number of oligosaccharide units attached to a particular secreted protein can be highly variable, resulting in a wide range of apparent molecular weights attributable to a single glycoprotein.

Murine IL-7 (mIL-7), simian IL-7 (sIL-7) and human IL-7 (hIL-7) are secreted glycoproteins and, in a first variant, the IL-7 conformer of this invention is glycosylated. The conformer may comprise various types of oligosaccharide units, depending on the production system and conditions. These may be, for instance, N-acetyl glucosamine, N-acetyl galactosamine, mannose, galactose, glucose, fucose, xylose, glucuronic acid, iduronic acid and/or sialic acids.

In a particular embodiment, the IL-7 conformer of this invention is moderately glycosylated. Preferably, the glycosylation is of the CHO type, even more preferably, produced by a CHO glycosylation mutant that stably expresses $\alpha 2,6$ sialyltransferase and presents a deficiency in CMP-Neu5Ac Hydrolase activity. Such glycosylation typically includes N-acetyl glucosamine, N-acetyl galactosamine, mannose, galactose, glucose, fucose, xylose, glucuronic acid, iduronic acid and/or sialic acids.

In an other, preferred, embodiment, the IL-7 conformer comprises a human type glycosylation. To produce such glycosylated form, the IL-7 conformer is typically produced by recombinant technology in a human host cell, which may be selected from HEK-293 (Human Embryonic Kidney), HER (Human Embryonic Retina), HEK (Human Epidermal Keratinocytes), human thymus, human bone marrow or human bone marrow stromal cell lines.

In an other, preferred variant, the IL-7 conformer is not glycosylated. Indeed, the invention shows that this conformer exhibits full biological activity in unglycosylated form.

A recombinant human IL-7 conformer having no carbohydrate, reduced carbohydrate or human carbohydrate pattern is highly preferred for therapeutic use.

In a specific embodiment, the invention relates to a specific IL-7 conformer, wherein said IL-7 conformer has the amino acid sequence of SEQ ID NO:4, comprises the disulfide bridges Cys2-Cys92; Cys34-Cys129 and Cys47- Cys141, and is human glycosylated, moderately glycosylated or unglycosylated. In an other embodiment, the sequence of the conformer comprises an additional methionine residue at the N-terminal end (SEQ ID NO:2) and is unglycosylated.

The purified conformer may be in the form of a monomer, or associated or complexed with a particular compound of choice. In this regard, in a particular embodiment, the IL-7 conformer is associated to the hepatocyte growth factor ("HGF"), as a heterodimer. The heterodimer may be obtained chemically, by complexation or by recombinant technology (i.e., by genetic fusion).

In an other particular embodiment, the IL-7 conformer is functionally attached to a Fc portion of an IgG heavy chain, typically through a peptide hinge region. Such fusion molecules have potentially increased stability and half-life in vivo. The IgG moiety is most preferably a human IgG1 or IgG4.

Drug Substance and Pharmaceutical compositions

The invention also relates to a drug substance comprising, as the desired product, an IL-7 conformer as described above, said drug substance being substantially free of IL-7 product-related substances and product related impurities.

A preferred drug substance is further substantially free of process related impurities.

Within the context of the present application, the term "drug substance" refers to a product suitable for use as the active principle of a medicament. The "drug substance" according to this invention is, by nature, a complex product, i.e., as a result of its production method (e.g., recombinant DNA technology).

The present invention now discloses that, in order to produce efficient therapeutic and vaccine enhancement effects, an IL-7 drug substance or pharmaceutical composition should contain, as the major molecular species, the conformer refolded according to the 1-4; 2-5; 3-6 disulfide bridges. Although other bioactive conformers, r-hIL-7 molecular variants, such as 1-6; 2-5; 3-4 do exist and have been described in the literature for long term use and/or repeated administrations, the results obtained by the inventors now demonstrate, for the first time, that these products will behave as product related impurities, presenting new epitopes and susceptible to induce a neutralizing immune response toward the preferred conformation of IL-7.

The present invention also discloses the fact that, in the specific case of a recombinant IL-7-containing pharmaceutical compositions, other potential product-related substances and product-related impurities, which would normally be included in the specification of the drug substance and/or drug product, although

5   bioactive, behave like new epitopes because of the potent vaccine enhancing property of IL-7. These products should thus be strictly minimized because they are able to trigger an immune reaction against the desired IL-7 molecule.

Bioactivity in a specific bioassay is a key critical result to qualify the activity of a

10   recombinant protein for therapeutic use (see ICH Q6B, Harmonized Tripartite Guideline which concern specifications, test procedures and acceptance criteria for biotechnological drug substances and products. Published in the Federal Register in August 18, 1999; issued as 64FR page 44928 (FDA) and adopted in Europe by the Committee for Proprietary Medicinal Products in March 1999; issued as

15   CPMP/ICH/365/96). Bioactivity is in fact the only single test of activity usually retained as a specification of activity for production batch release. Compliance with bioactivity specifications usually warrants that the recombinant protein drug substance / drug product is active in humans. Although various other *in vivo* animal models are implemented to test the activity of recombinant proteins, since the

20   heterologous human recombinant proteins differ from the homologous protein in the tested animal species, the evaluation of long term activity in animals is hampered.

The previous experience of long term use of recombinant proteins in humans shows that the administration of an unmodified protein sequence, devoid of highly

25   immunoreactive "process related" contaminants such as DNA, endotoxins and host cell proteins, allows a long term activity and benefit without neutralizing immune reactions. Product related substances, which are molecular variants of the desired product (as defined in ICH Q6B guideline), and product related impurities, as long as they have a significant bioactivity, are tolerated at a relatively high percentage of

the drug substance / drug product composition. ICH international guideline Q6B stipulates that "*product related substances are molecular variants of the desired product formed during manufacture and/or storage, which are active and have no deleterious effect on the safety and efficacy of the drug product. These variants*
5 *possess properties comparable to the desired product and are not considered impurities*". In that light, various product related substances and/or product related impurities, molecular variants of IL-7, if bioactive, would authorize a batch release based on the bioassay. The present invention now discloses that, in contradiction with these guidelines and common practice, in the specific case of IL-7, these
10 molecular variants, whatever their bioactivity, would induce an immune response against the r-hIL-7 desired product and/or endogenous human IL-7 and neutralize the long term therapeutic benefit of the recombinant protein. This is mainly due to the association of these molecular variants, behaving like new epitopes, with the potent vaccine enhancing capacity of IL-7. Among these molecular variants, the
15 present invention now discloses that a specific attention should be paid to conformers resulting from an inappropriate refolding of the molecule, to deamidated forms, to dimers and to hyperglycosylated forms, for example, and more generally to molecular bioactive variants susceptible to induce an immune reaction because they differ from the preferred, specifically identified, molecular species.
20

In particular, by conducting *in vivo* experiments in monkey (as reported in the examples), the inventors have been able to demonstrate that efficient drug substances would require, as a major constituent, an IL-7 conformer as described above, and should also be substantially free of other conformers or product-related
25 impurities. Such results would not have been achieved in *in vitro* bioassay, where all compounds are bio-active, or in *in vivo* assays in rodents, where IL-7 has a very strong lymphopoietic effect on B cells. By cloning the simian IL-7 and conducting *in vivo* experiments in monkeys using either drug substances of this invention or complex products as disclosed in the prior art, the inventors have unexpectedly

demonstrated that a specific conformer was active and needed to be used in purified form.

A particular object of this invention thus resides in a drug substance comprising, as the desired product, an IL-7 conformer as described above, said drug substance being substantially free of IL-7 product-related substances and product related impurities.

The term "substantially free", as used herein, indicates that the drug substance contains no significant or adverse amount of product-related substances, product related impurities and process-related impurities. More specifically, the drug substance should contain less than 5%, more preferably less than 3%, even more preferably less than 2% of product-related substances, product related impurities and process-related impurities. Most preferred drug substances contain less than about 1% of product-related substances and/or product related impurities and only trace amount of process-related impurities.

IL-7 product-related substances designate IL-7 molecular variants, which include, for example, other IL-7 conformer and/or any active or inactive peptide or polypeptide fragments of IL-7.

IL-7-related impurities include, for example, human IL-7 polypeptides comprising mono or bi-disulfide bridges, contiguous disulfide bridges and any disulfide bridges combination such as the following: Cys: 1-6; 2-5; 3-4, and adjacent disulfide bridges: Cys: 1-2; 3-4; 5-6. Other IL-7 related impurities include aberrant glycosylated form including hyper-glycosylated IL-7, yeast-glycosylated IL-7, truncated IL-7, deamidated recombinant IL-7, dimeric or multimeric protein comprising IL-7, oxidized methionine form or a combination thereof.

Whatever their biological activity, these IL-7 molecular variants and IL-7-related impurities should be strictly minimized or discarded.

Process related impurities include DNA, endotoxins, cell debris, etc.

Such impurities as mentioned above are often generated by recombinant DNA processes. However, their impact on the final product activity has not always been assessed and efficient methods for their removal are not always available. The present invention now demonstrates that conventional recombinant methods of producing IL-7 also generate various impurities as described above. The invention more particularly demonstrates that various IL-7 molecular variants are present and that various amino acid sequences are present. Furthermore, the inventors have discovered that the most powerful mid or long term *in vivo* biological activity of r-IL-7 is carried by a specific conformer having three particular disulfide bridges: Cys: 1-4 (Cys2-Cys92); 2-5 (Cys34-Cys129); 3-6 (Cys47-Cys141). Missing or improper disulfide bridges could very significantly decreases IL-7 activity. Furthermore, the presence of such impurities produces molecules which are immunoreactive upon administration to human or non-human primates, and thus impair the therapeutic benefit of the IL-7 conformer of the present invention.

A preferred drug substance is thus a drug substance wherein the total amount by weight of IL-7 comprises at least 95% by weight, preferably at least 98% by weight, more preferably at least 99.5% by weight of an IL-7 conformer according to the invention.

The invention also relates to a pharmaceutical composition comprising an effective amount of a drug substance as described above and one or more pharmaceutically compatible or acceptable carriers, excipients or diluents.

The invention shows that pharmaceutical compositions comprising a substantially pure IL-7 conformer as described above clearly increase the vaccine properties of IL-7 and its capacity to stimulate antigen-specific immune responses. The invention thus demonstrates that highly efficient biological preparations are obtained when IL-7 related impurities are removed and a specific conformer as described above is used.

The pharmaceutically compatible or physiologically acceptable carrier, excipient or diluent may be selected from neutral to slightly acidic, isotonic, buffered saline, solutions or suspensions and more preferably from sucrose, trehalose, and amino acid. The pharmaceutically compatible carrier is preferably contained in an appropriate buffer to form an isotonic solution. Numerous molecular variants of IL-7 such as hyperglycosylated recombinant proteins produced from recombined yeast strains, IL-7 dimers produced at low ionic strength, and deamidated forms, although bioactive should be strictly minimized. During manufacture and storage, exposition of the molecule to high or moderately high pHs: 8 to 10 triggers the formation of deamidated forms. Storage at pH 8.5 to 9 for a few days at room temperature induces appearance of these deamidated molecular species. Steps including high pHs are preferably minimized during the process and stability of the recombined protein is improved if stored in a slightly acidic buffer pHs 5 to 7. The appropriate buffer has preferably a pH range comprised between 5 to 7.5, preferably 6 to 7, even more preferably of about 6.5 and is preferably an organic salt selected from a sodium citrate buffer or an ammonium acetate buffer. The pharmaceutical composition may be in the form of a suspension, solution, gel, powder, solid, etc. The composition is preferably a lyophilized form. Indeed, product can be formulated as a lyophilisate using appropriate excipient solutions (e.g., sucrose and/or trehalose in a range of 1/1 to 10/1, preferably 2/1 to 6/1 sugar/drug substance mass ratio for example) as diluents and lyo-protectors.

The composition may comprise stabilizing agents, such as sugar, amino acids, etc. The composition may comprise any saline solution, including phosphates, chloride, etc. Appropriate dosages can be determined in trials.

5   Actual dosage levels of active ingredients in the compositions of the present invention .may be adapted so as to obtain an amount of active ingredient that is effective to obtain a desired therapeutic response for a particular composition and method of administration. The selected dosage level therefore depends upon the desired therapeutic effect, the route of administration, the desired duration of

10   treatment and other factors. Generally, dosages of 3 to 300 µg/kg , preferably, 10 to 100 µg/kg , administered by subcutaneous route, can be expected to induce a biological effect preferably while administered from once daily down to twice weekly. It should be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the body weight, general

15   health, sex, diet, time and route of administration, rates of absorption and excretion, combination with other drugs and the severity of the particular disease being treated. In the specific case of IL-7 a particular attention should be paid to the immune status of the patient before adjusting dose level. The more the patient is immuno-depressed, as judged for instance through peripheral CD4 T-Cell counts,

20   the less the dose necessary to induce a relative increase in lymphocyte counts. In severely immuno-depressed primates, sub-cutaneous doses of 60 µg/kg daily produce a strong increase in T-Cell counts (X3 to X5, in a dose-dependent manner), but in primates with normal lymphocyte counts, higher doses such as 300 µg/kg are necessary to increase lymphocyte counts from 2 to 5-fold. After sub-

25   cutaneous administration, recombinant IL-7 has a surprisingly long blood half life in primates. Its effect on cell cycling lasts for 24 to 48 hours, allowing efficient treatment schedules with single injection daily down to two injections weekly.

Pharmaceutical compositions according to the invention are preferably administered from once daily down to twice weekly, only in order to obtain and/or stimulate patients immune regeneration.

5   Preferred administration routes are parenteral routes. The parenteral route is preferably an intra-tumoral, more preferably an intra-venous or a sub-cutaneous administration. It includes also intra-arterial, intra-peritoneal or intra-muscular injections. It should be understood, however, that any other suitable administration route may be contemplated depending upon the health status and the reactivity of
10 the patient.

The pharmaceutical composition may comprise additional active ingredients, such as immuno-stimulating agents, preferably selected from a hematopoietic cell growth factor, a cytokine, an antigenic molecule (or antigen) and an adjuvant, for
15 combined, separate or sequential use.

Such additional active ingredients may be formulated in combination with the IL-7, or, separately, for combined, separate or sequential use. In a first variant, the active ingredients are formulated together, in the same recipient or vessel. In an other,
20 preferred variant, they are conditioned separately, i.e., in distinct vessels or recipients. According to this embodiment, the ingredients may be administered separately, e.g., simultaneously or sequentially (e.g., at different injection sites or at different time points), to produce the most efficient biological effect. Also, as mentioned above, repeated administrations of one or the two active ingredients may
25 be performed.

In this respect, the invention relates to a pharmaceutical composition comprising a substantially pure IL-7 conformer as described above and an active ingredient

selected from an immuno-stimulant and an antigenic molecule, for combined, separate or sequential use. Adjuvants are preferably formulated separately.

The hematopoietic cell growth factor is preferably selected from the Stem Cell
5 Factor (SCF), particularly the soluble form of the SCF, G-CSF, GM-CSF, Flt-3 ligand, IL-15 and IL-2. Typical examples of cytokines for vaccine enhancement include cytokines that induce and/or stimulate a Th1-type immune response. The cytokine is preferably selected from γ interferon, IL-2, IL-12, RANTES, B7-1, MIP-2 and MIP-1α. It should be understood that other factors, interleukins and/or
10 hormones may be used in combination with IL-7 to provide additional therapeutic benefit.

A specific composition of this invention comprises a substantially pure IL-7 conformer as described above and Stem Cell Factor, particularly the soluble form
15 thereof, IL-15 and/or Flt-3 ligand.
An other specific composition of this invention comprises a substantially pure IL-7 conformer as described above and a cytokine selected from γ interferon, IL-2, IL-12, RANTES and MIP-1α.
An other specific composition of this invention comprises a substantially pure IL-7
20 conformer as described above, a Stem Cell Factor and a cytokine.

As indicated above, the pharmaceutical composition may further comprise one or several antigens (or antigenic molecules), for combined, separate or sequential use. The antigen may be any synthetic or natural peptide, a recombinant protein, a
25 killed, inactivated or attenuated pathogen product, a microorganism, a parasite, a lipid, etc., a portion thereof and a combination thereof. The antigen may be an entire protein, or any epitope-containing fragment or portion thereof, particularly peptides that are presented to the immune system through MHC class I or MHC class II molecules. The antigen can be any viral antigen, bacterial antigen, parasite

antigen, tumor antigen, etc. Specific examples of antigens include antigens derived from HIV, Varicella Zoster virus, Influenza virus, Epstein Barr virus, type I or 2 Herpes Simplex virus, human cytomegalovirus, Dengue virus, Hepatite A, B, C or E virus, Syncytium respiratory virus, human papilloma virus, mycobacterium
5   tuberculosis, Toxoplasma and Chlamydia.

A particular object of this invention relates to a composition comprising a substantially pure IL-7 conformer as described above and an antigenic molecule, for combined, separate or sequential use. The composition may further comprise one
10   or several immuno-stimulating agents as disclosed above, for combined, separate or sequential use.

A further particular object of the present invention concerns a pharmaceutical composition comprising an IL-7 conformer as described above, wherein said
15   pharmaceutical composition is administered simultaneously, a few days before or sequentially with one or several antigenic molecules in order to obtain and/or stimulate an antigen-specific immune response in a subject.

A further particular object of the present invention concerns a method of causing or
20   enhancing an antigen-specific immune response in a subject, comprising administering to a subject said antigen (or an epitope-containing fragment thereof) and a pharmaceutical composition comprising an IL-7 conformer as described above. The pharmaceutical composition may be administered simultaneously, a few days before or sequentially with said antigen in order to obtain and/or stimulate an
25   antigen-specific immune response in a subject.

In another preferred embodiment, the composition of the invention further comprises an adjuvant. The adjuvant may be selected from any substance, mixture, solute or composition facilitating or increasing the immunogenicity of an antigen and

able to induce a Th1-type immune response, such as CpG, QS21 and monophosphoryl lipid A. Such adjuvants are particularly suited to produce and/or amplify a specific immune response against antigen(s) in mammalian subjects, particularly in humans. The adjuvant is preferably conditioned and administered

5   separately from the IL-7 containing composition and/or at a distinct site of injection, preferably with the desired antigen(s).

The present invention also concerns a pharmaceutical composition comprising an effective amount of a human IL-7 conformer according to the invention in admixture

10   with a suitable diluent, excipient or carrier, for parenteral administration to a human patient for prophylactic or therapeutic stimulation of B or T lymphocyte development and proliferation, or for augmentation of an immune response. The pharmaceutical compositions of the invention induce a prolonged lymphopoiesis stimulation and/or amplified immune responses.

15

A pharmaceutical composition according to the invention may also be used in a human patient for prophylactic or therapeutic stimulation of B or T lymphocyte development and proliferation, for enhancement of global and/or specific immuno-reconstitution, or for enhancement of humoral and/or cellular immune responses.

20

A particular pharmaceutical composition according to the invention is for use to prevent or reduce opportunistic infections in immunodeficient patients.

Another particular pharmaceutical composition according to the invention is for use

25   to prolong lymphopoiesis stimulation and/or to produce specific immune response not only against dominant epitopes but also against sub-dominant or less immunogenic epitopes, epitopes having a lower affinity for the T cell receptor, which will allow to broaden the repertoire of a specific immune response in human patients.

The invention is particularly suited to produce a preventive or curative immune response in subjects, such as immunodeficient patients, cancer patients, patients undergoing grafts, patients infected with a virus or a parasite, elderly patients or any patients having low CD4 count etc.

Production methods and tools

An other aspect of the present invention is to provide appropriate constructs and methods for producing the above compositions, particularly the above IL-7 conformer and drug substances, in sufficient quantities and quality for pharmaceutical use thereof.

In this regard, the invention now discloses nucleic acid molecules comprising a sequence encoding an IL-7 polypeptide, said sequence being optimized for the expression, in a recombinant host, of biologically active IL-7, particularly of an IL-7 conformer as described above. More specifically, the nucleic acid molecules provide for a limited expression of truncated IL-7 polypeptides and increase the yields of production of biologically active human IL-7 conformer.

The human and simian IL-7-encoding DNA sequence (genomic or cDNA) contain, at position 49 after the "ATG" initiation codon, a second putative "ATG" which could behave as a second initiation codon in E. Coli. Indeed, this second "ATG" is preceded by a "pseudo Shine-Dalgarno" sequence (ribosome binding sequence). In order to create optimized products, the applicants have undertaken to inactivate this putative second initiation codon, thereby significantly reducing the potential production of an amino-terminal truncated form of r-hIL-7. To produce such optimized sequences, nucleotides in the coding sequence were mutated in order to

inactivate the "SD-like" sequence, without modifying the resulting, encoded, r-IL-7 amino acid sequence.

A further object of the invention thus relates to nucleic acid molecules encoding a
5 human IL-7 polypeptide, wherein said nucleic acid contains a modified sequence avoiding the production of an amino-terminal truncated polypeptide, more specifically by inactivation of the "SD-like" sequence (i.e., of the putative second initiation codon located at position 49 in the sequence, which encodes methionine residue 18 in SEQ ID NO: 2).
10

Inactivation of the putative second initiation codon may be achieved, more preferably, by modifying the sequence in at least one of the 5 codons preceding the ATG codon encoding methionine 18 in SEQ ID NO: 1, i.e., in codons 13-17 encoding amino acid sequence TyrGluSerValLeu. In a specific embodiment, the
15 sequence is modified by altering the codon coding for a serine at position 15 in SEQ ID NO: 1 or 2. More specifically, in a preferred sequence, this codon is modified so as not to contain an AG doublet. Suitable codons may be selected, for instance, from TCC, TCT, TCA and TCG. In SEQ ID Nos: 1 and 3, the TCC codon has been used. The codon encoding Tyr at position 13 (based on the numbering in SEQ ID
20 NO:1) may be selected from TAC or TAT. The codon encoding Glu at position 14 (based on the numbering in SEQ ID NO:1) may be selected from GAG and GAA. The codon encoding Val at position 16 (based on the numbering in SEQ ID NO:1) may be selected from GTT, GTC, GTA and GTG. Finally, the codon encoding Leu at position 17 (based on the numbering in SEQ ID NO:1) may be selected from
25 CTG, CTA, CTT, CTC, TTA and TTG. All combinations of the above indicated codons are possible. A specific example of an altered DS-like sequence corresponds to nucleotides 37-51 of SEQ ID NO:1.

A specific, preferred nucleic acid molecule of this invention comprises the sequence of SEQ ID NO:1 and codes for a recombinant human IL-7 polypeptide.

Another specific, preferred embodiment of the invention relates to a nucleic acid
5   molecule as described above which further comprises a signal sequence causing secretion of the produced polypeptide. The signal sequence may be selected from the natural signal sequence of the human IL-7 protein, or from any heterologous signal sequence. Such sequences may originate from other secreted proteins, such as the human growth hormone, or be artificial or synthetic. A preferred signal
10   peptide may be a natural signal peptide of a human growth factor or of a human growth hormone and, more preferably, a natural signal peptide of the human IL-7. Preferred sequences are those functional in competent mammalian host cells. A specific example of such an improved sequence comprises SEQ ID NO:3. This sequence comprises a leader sequence and an inactivated SD-like sequence, for
15   improved expression.

Another object of the invention concerns a polypeptide encoded by a nucleic acid sequence as described above, which may be glycosylated or unglycosylated.

20   A preferred object of the invention concerns a polypeptide, as described above, wherein the tertiary structure of said polypeptide comprises the following three disulfide bridges: Cys: 1-4; 2-5; 3-6.

The present invention further provides vectors comprising a nucleic acid as
25   described above, as well as recombinant host cells comprising said nucleic acid or said vectors. The nucleic acids and vectors may be used to produce recombinant human IL-7 polypeptides in various competent host cells, as well as for gene therapy purposes.

The vector may be a plasmid, virus, phage, cosmid, episome, etc. Preferred vectors are plasmids, which can be produced based on commercially available backbones, such as pBR, pcDNA, pUC, pET, etc. The vector typically comprises regulatory elements or sequences to control or mediate expression of an IL-7 polypeptide from

5 the optimized coding nucleic acid. The regulatory sequences may be chosen from promoters, enhancers, tissue-specific signals, peptide signals, introns, terminators, polyA sequences, etc. Such regulatory elements or sequences may be derived from mammalian, bacterial, yeast, bacteriophage or viral genes, or from artificial sources. Useful promoters for prokaryote expression (such as *E. coli*) include T7 RNA

10 polymerase promoter (pT7), TAC promoter (pTAC), Trp promoter, Lac promoter, Tre promoter, PhoA promoter for example. Suitable promoters for expression in mammalian cells include viral promoters (e.g., CMV, LTR, RSV, SV40, TK, pCAG etc), domestic gene promoters, etc.

15 The vector may further comprise an origin of replication and/or a marker gene, which may be selected from conventional sequences.

The present invention thus also provides recombinant host cells comprising a nucleic acid or a vector as described above. The host cell may be selected from any

20 eukaryotic and prokaryotic cells, typically from a mammalian cell (in particular a human, rodent, canine cell), a bacterial cell (in particular E. coli, Bacillus Brevis, Bacillus Subtilis), a yeast cell, a plant cell and an insect cell. Where yeast, plant or insect cells are used for production purposes, the products obtained in said cells are preferably submitted to a treatment step in order to produce unglycosylated

25 products. These host cells may be adapted to serum-free media. Production may also be accomplished in a transgenic animal.

Preferred recombinant host cells are selected from a mammalian cell, in particular a human cell, and a bacterial cell, as well as derivatives or mutants thereof.

Specific examples of suitable host cells include bacteria, which provide for non-glycosylated production of proteins. In Bacteria (e.g., *Escherichia coli*), recombinant IL-7 is generally expressed as inclusion bodies. This host is particularly advantageous and may be used, more specifically, with a vector comprising a

5   sequence SEQ ID NO:1.

Other examples of suitable host cells include mammalian cells, which provide for glycosylated production of proteins. One may cite Chinese Hamster Ovary (CHO) cells, Baby Hamster Kidney (BHK) cells, Human Embryonic Kidney (HEK-293) cells, etc. In such mammalian cells, IL-7 may be produced as a secreted protein using

10   functional signal peptide sequences. These hosts may be used, more specifically, with a vector comprising a sequence SEQ ID NO:3.

A specific object of this invention is a prokaryotic host cell comprising a nucleic acid molecule comprising SEQ ID NO: 1.

15   Another specific object of this invention is a eukaryotic host cell comprising a nucleic acid molecule comprising SEQ ID NO: 3 or SEQ ID NO: 12.

Another specific object of this invention is a eukaryotic or prokaryotic host cell comprising a nucleic acid molecule encoding an IL-7 polypeptide comprising amino acids 26-152 of SEQ ID NO: 13.

20

A further object of the present invention relates to antibodies immunoreactive with an IL-7 conformer as described above. Such antibodies may be produced according to conventional methods, including immunization of animals and collecting the serum (polyclonal) or preparing hybridomas from spleen cells (monoclonal).

25   Fragments (e.g., Fab') or derivatives of antibodies (e.g., ScFv) may be produced by known biological and chemical methods. Preferred antibodies are specifically immunoreactive with an IL-7 conformer as described above, i.e., can bind the IL-7 conformer without substantially binding IL-7 polypeptides which do not comprise the following three di-sulfide bridges: Cys2-Cys92, Cys34-Cys129 and Cys47-Cys141.

Although non-specific or less effective binding to such other antigens may be observed, such non-specific binding can be distinguished from specific binding to the particular conformer of this invention.

5　The antibody is preferably of simian, murine or human origin or has been humanized.

The invention also relates to a hybridoma cell line that produces a monoclonal antibody as described above.

10　Such antibodies are useful in detecting IL-7 conformer or in neutralizing IL-7 biological activity in assays or experiments involving multiple lymphokines. A composition suitable for diagnosis, assay or therapy comprising such monoclonal antibodies is also an object of the present invention.

15　The invention also provides a method of producing or amplifying an optimized IL-7 nucleic acid from a sample, the method comprising:

i)　providing a sample containing an IL-7 gene or coding sequence,

ii)　contacting said sample with a pair of primers that produce an optimized sequence, particularly a sequence devoid of a functional second initiation

20　codon at position 49, and

iii)　producing or amplifying the optimized IL-7 nucleic acid.

The sample may comprise genomic DNA, cDNA or RNA.

Amplification of the gene may be produced by any technique known per se, such as
25　PCR, RT-PCR, or other amplification techniques. Amplification usually requires the use of a pair of primers characterized in that the forward sequence hybridizes with the 5' end of the DNA and in that the reverse sequence hybridizes with the 3' end of the same DNA. Specific examples of primers are as follows :

Pair 1: SEQ ID NO: 5 and SEQ ID NO: 6,

Pair 2: SEQ ID NO: 7 and SEQ ID NO:6, and

Pair 3: SEQ ID NO: 8 and SEQ ID NO: 6

Such particular nucleic acid primers represent further objects of this invention (e.g., SEQ ID NO:5-8).

5　It should be understood that other primers can be designed by the skilled artisan, such as fragment of the optimized IL-7 gene, for use in the amplification step and especially a pair of primers comprising a forward sequence and a reverse sequence, wherein said primers of said pair hybridize with a region of the mutated IL-7 gene and allow amplification of at least a portion of the IL-7 gene.

10

Another object of the present invention relates to processes which can be used, on an industrial scale, for the production of a pharmaceutical grade, substantially pure IL-7 conformer as described above. The process leads to high yields of recombinant IL-7 conformer suitable for therapeutic use. The invention also

15　provides novel methods of controlling IL-7-containing compositions, to determine the presence of amount of an IL-7 conformer as described above.

In a particular aspect, the method of producing an IL-7 conformer or a drug substance as defined above comprises:

20　a)　providing a sample comprising IL-7 polypeptides, and

b)　purifying an IL-7 conformer as described above.

The sample used in step a) may be any biological sample containing an IL-7 polypeptide. This includes, for instance, a cell supernatant, a biological fluid, a cell

25　extract, a tissue sample, etc. Preferred samples are cell supernatants of recombinant host cells that produce a recombinant IL-7 polypeptide, more preferably a recombinant human or simian IL-7 polypeptide, even more preferably, a recombinant human IL-7 polypeptide.

Most preferred samples are (obtained from) cell supernatants of recombinant host cells that contain an optimized IL-7 coding nucleic acid molecule as described above, more particularly a polynucleotide comprising SEQ ID NO: 1 or 3 or 12.

5   The sample may be subjected to various treatments or conditions in order to increase purity of IL-7, to release IL-7 from inclusion bodies, to remove cell debris or other particular bodies, etc. Typical examples of such treatments include centrifugation, filtration and/or clarification. The sample may thus be enriched for IL- 7 polypeptide.

10

To increase the yields or efficiency of the method, it is highly desirable to produce a sample containing or enriched in correctly folded IL-7 polypeptides.

In this regard, where the sample is (or derives from) a culture of prokaryotic host
15   cells encoding an IL-7 polypeptide, particular treatments of the sample are necessary. Such treatments include:

   i) treating said sample to cause a complete denaturation of said IL-7 polypeptides,

   ii) optionally purifying the denatured polypeptide obtained in step i) and

20   iii) refolding the polypeptides.

   Step i) comprises the treatment of the sample to cause denaturation of IL-7 polypeptides. Denaturation designates a destruction and reduction of all tertiary structures in the polypeptide, such as disulfide bridges or other intra-molecular
25   bounds. Denaturating treatment comprises the dissolution of inclusion bodies in a denaturant buffer to reach a fully denatured, solubilized protein. Denaturation may be obtained with a buffer comprising guanidine hydrochloride, or urea, EDTA, β mercaptoethanol or dithiotreitol (DTT). Appropriate dosages can be determined by persons skilled in the art and depend on the level of denaturation required. Other

denaturing conditions, known in the art, may be applied as well, alone or in combination. A preferred level of denaturation corresponds to the destruction of all the disulfide bridges of IL-7. A preferred full denaturation protocol uses 8 M guanidine hydrochloride buffer. High guanidine hydrochloride molarity is preferred to allow full denaturation of the protein prior to its correct refolding. This stage of denaturation may be controlled by analytical techniques known in the art, such as electrophoresis, for instance.

Purification step ii) may be performed by different techniques known per se, but which have not been used so far in the present combination to produce a fully active IL-7 polypeptide conformer. These techniques are more preferably selected from hydrophobic interaction chromatography, ion exchange chromatography, affinity chromatography and gel filtration chromatography, either alone or in various combinations. Such methods allow to remove DNA and other impurities (lipids, etc.) which would lower recovery of the subsequent refolding step. In a preferred embodiment, step ii) comprises a hydrophobic interaction chromatography step. Such chromatography may be carried out using various supports and formats, preferably using HIC butyl, preferably in denaturing conditions. To perform this step, a preferred pH range is comprised between 6 and 9, preferably between 7 and 8,5 inclusive. Step ii) may be carried out on any support, preferably on batch or in column using an appropriate gel.

The refolding step iii) comprises a renaturation and preferably reoxydation of the IL-7 polypeptides and, typically, a further desalting step performed by filtration (for example a gel filtration) or ultrafiltration. In a preferred embodiment, the refolding comprises an ion exchange chromotography step to separate the active new conformer from the unwanted forms. In a most preferred embodiment, the refolding comprises a step of contacting the purified polypeptides with a selected affinity chromatography support in order to separate the active new conformer from the

unwanted forms. This very advantageous step is selective for the elution of the correctly refolded IL-7.

In a particular embodiment of the invention, the refolding step iii) comprises passing the solution obtained in step ii) through a column comprising polymers of sulfated polysaccharides. The sulfated polysaccharide is preferably dextran sulfate or heparin and the matrices can be sepharose, acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. A preferred matrix is heparin sepharose. The process may further comprise an additional step of eluting the desired IL-7 conformer.

In a particular embodiment, the above affinity column may be used both to purify and refold the polypeptides, i.e., to perform simultaneously steps ii) and iii).

Where the sample is (or derives from) a culture of eukaryotic host cells encoding IL-7 polypeptides, it may not be necessary to perform the above treatment steps i) to iii). Indeed, in such a situation, the sample may comprise correctly folded IL-7 proteins, although in complex admixture with other product-related substances and impurities. This is particularly true where the recombinant eukaryotic host cell comprises a nucleic acid molecule causing expression and secretion of the IL-7 polypeptides in the culture medium.

In this case, the sample may be directly subjected to the purification step b).

Purification step b) may comprise one or several purification steps such as ion exchange chromatography, filtration steps, affinity chromatography, hydrophobic interaction chromatography etc., in order to eliminate drug related substances such as other conformers, deamidated forms, dimers of the protein, residual impurities, including DNA, endotoxins, etc.

In a preferred embodiment, the purification step b) comprises loading the sample through a column packed with a specific gel comprising sulfated polysaccharides immobilized on a resin (dextran sulfate or heparin for example).

In another particular embodiment the purifying step comprises loading the sample through a column packed with a specific gel comprising a monoclonal antibody immobilized on a resin (dextran sulfate or heparin for example).

These methods allow the reproducible and efficient production of a substantially pure fully active human IL-7 conformer as described above. The methods are particularly advantageous since the recombinant IL-7 conformer can be obtained with a purity of at least 95% by weight, preferably at least 98% by weight with respect to the total amount of IL-7. Furthermore, the above purification method may be applied to polypeptides other than human IL-7, particularly to other cytokines having one or several disulfide bridges, including animal IL-7 (e.g., monkey IL-7), IL-13, IL-15, etc.

Each step of the above described process may be controlled by analytical methods, including SDS-PAGE analysis. The primary structure of the optimized IL-7 may be controlled and characterized by determining the gene and/or amino acid sequence, by peptide mapping analysis, after tryptic digestion, by determining molecular weight with SDS PAGE, SEC, MALDI TOF and/or Mass spectometry, by determining hydrophobicity with reverse phase HPLC for example, and/or by determining the electric charge with cation exchange chromatography HPLC or isoelectrofocalisation analysis for example.

In a specific variant, the invention relates to a method of producing an IL-7 conformer as defined above, the method comprising at least the following steps:

a) providing a sample comprising IL-7 polypeptides produced by a recombinant prokaryotic host cell,

a') treating said sample to cause a complete denaturation of said IL-7 polypeptides,

a") optionally purifying the denatured polypeptide obtained in step a"),

a''') refolding the polypeptides, and

b) purifying the polypeptide to produce a conformer as described above.

In an other specific variant, the invention relates to a method of producing an IL-7 conformer as defined above, the method comprising at least the following steps:

a) providing a sample produced by a recombinant eukaryotic host cell, said sample comprising a refolded IL-7 polypeptide, and

b) implementing one or several purification steps in order to eliminate product related substances or impurities.

In a preferred embodiment, the production methods of this invention comprise an additional step c) of characterizing and measuring or quantifying the particular IL-7 conformer as disclosed above contained in the resulting product. The physical and biological characterization of the desired IL-7 conformer may be obtained by Mass spectrometry (MALDI-TOF), infra-red spectroscopy, nuclear magnetic resonance (NMR), by determining circular dichroïsm, by measuring the affinity toward a specific monoclonal antibody raised against said IL-7 conformer, or heparin affinity chromatography. Once characterized, the quantification of said conformer may be performed by ELISA, bioassay, affinity of said IL-7 conformer for IL-7 receptor and any method of protein quantification if applied to the isolated conformer.

In this regard, the invention also provides and concerns a method to identify and/or measure the quantity of IL-7 conformer and/or related impurities in a sample, particularly in a pharmaceutical preparation. Such characterization methods can be

used to initially characterize and qualify the protein for filing a therapeutic use, in quality control of pharmaceutical batches. Indeed, the present invention now demonstrates that a fully biologically active IL-7 conformer exists and that the presence of impurities such as other conformers or IL7 peptide fragments may generate highly undesirable side effects. The invention thus proposes, for the first time, a method of characterizing and controlling IL-7-containing preparations, to determine the presence and/or relative quantity of the specific IL-7 conformer of this invention. Preferred methods use western blot, size-exclusion HPLC, ECP assay, bacterial endotoxins assay, Limulus Ameobocyte Lysate test (LAL test), DNA quantification, SDS-PAGE, reverse phase HPLC, ion exchange HPLC, heparin Affinity HPLC, BCA method, ELISA, UV absorption and/or a Bioassay. These methods may be carried out alone or in various combinations.

The invention also provides a method of producing an IL-7 drug substance or pharmaceutical composition, said method comprising (i) culturing a recombinant host cell encoding an IL-7 polypeptide, (ii) isolating said recombinant polypeptide to produce an IL-7 drug substance and (iii) conditioning said IL-7 drug substance to produce a pharmaceutical composition suitable for therapeutic or vaccine use, said method further comprising a step of identifying, characterizing or measuring, in said drug substance or pharmaceutical composition, the quantity and/or quality of an IL-7 conformer as defined above and, more preferably, a step of selecting the drug substance or pharmaceutical composition which comprises, as the active ingredient, more than about 95% of said IL-7 conformer.

The characterizing step may be carried out by a variety of techniques, more preferably by mass spectrometry-related methods, with or without tryptic digest, circular dichroism, NMR, specific monoclonal antibody analysis for disulfide bridges and/or conformation characterization. The identification of molecular variants and product related impurities is preferably performed by using one or several methods

selected from bi-dimensional electrophoresis, isoelectric focusing and ion-exchange chromatography for deamidated forms, size exclusion chromatography and SDS-PAGE analysis for dimeric or multimeric forms, and HPLC reverse phase with or without predigest for various forms including truncated forms and oxidized

5   methionine forms.

The step is particularly suited for quality control of clinical or pharmaceutical compositions, whereby only compositions comprising more than about 95% of the above IL-7 conformer are retained, preferably more than about 96%, 98% or 99,5%.

10

Another object of the present invention relates to the use of the isolated IL-7 polypeptide conformer or of a recombinant IL-7 conformer obtained with the processes as described above, for the manufacture of a pharmaceutical composition to prevent or treat a disease associated with an immunodeficiency,

15   particularly to induce a prolonged lymphopoiesis stimulation, to cause and/or amplify an immune response, particularly an antigen-specific immune response.

A further object of the invention relates to the use of an IL-7 conformer as a tool for experimental and pharmacological use in monkeys.

20

The invention also relates to kits for implementing the above methods comprising at least a primer specific for the optimized IL-7 gene and, optionally, a pair of primers and/or a probe and/or reagents for a nucleic acid amplification reaction and/or antibodies, as described above. Kits may alternatively comprise reagents to

25   produce an IL-7 polypeptide as described above, such as an optimized nucleic acid, a vector, a recombinant host cell, and/or protocols or reagents for the purification or quality control of such preparations.

Other aspects and advantages of the present invention will be described in the following examples, which should be regarded as illustrative and not limiting the scope of the present application.

5

EXAMPLES

Example A. Construction and Expression of optimized human (h) and simian (s) IL-7-coding nucleotide sequences:

10

A1.   *E. Coli* Expression (*E. coli* JM101):

1.1.   Construction of human IL-7-coding nucleotide sequence:

15   The human IL-7 encoding cDNA sequence was amplified by polymerase chain reaction (PCR) (Mullis et al.; 1987; Methods in Enzymology; 155:335-350) from human placenta cDNA (BioChain Inc.) using the following specific oligonucleotide primers which contain restriction endonuclease recognition sequences:

20   - SEQ ID NO: 5: IL75'

5'ATTC<u>CATATG</u>GATTGTGATATTGAAGGTAAAGATGGC3'

N*de*I

- SEQ ID NO: 6: IL73'

25   5'AGCC<u>GGATCC</u>TTATCAGTGTTCTTTAGTGCCCATCA3'

Bam*H*I

The human IL-7-encoding DNA sequence presents, at position 49 after the "ATG" initiation codon, a second putative "ATG" which could behave as a second initiation

30   codon in *E. Coli*, since this second "ATG" is preceded by a "pseudo Shine-

Dalgarno" sequence (ribosome binding sequence). To avoid the potential production of an amino-terminal truncated form of r-hIL-7, some of the nucleotides of the "SD-like" sequence were mutated (without modifying the resulting encoded r-hIL-7 amino acid sequence), thereby producing an improved methionyl-IL-7-coding-

5 DNA sequence containing one or more preferred codon(s) for expression in *E. Coli* cells.

The suppression of the SD-like sequence in the IL-7-encoding DNA sequence was performed by site directed mutagenesis PCR, using the following oligonucleotide

10 primers:

- SEQ ID NO: 7: mutIL75'

5'TAGGGAATTC<u>CATATG</u>GATTGTGATATTGAAGGTAAAGATGGCAAACAATACGA

        N*de*I

15 GTCCGTTCTG3'

- SEQ ID NO: 6: IL73'

        5'AGCC<u>GGATCC</u>TTATCAGTGTTCTTTAGTGCCCATCA3'

        B*am*HI

20

The amplification and suppression of the SD-like sequence may be realized by single step using oligonucleotide primers above.

PCR products were assayed by polyacrylamide or Agarose gel electrophoresis in

25 the presence of ethidium bromide and visualization by fluorescence of DNA bands stimulated by UV irradiation. A single major product band of the size corresponding to the IL-7 PCR fragment was isolated and inserted into the plasmid vector pCR II-TOPO (Invitrogen) using the TA-cloning method. The ligation products were transformed into TOP10 competent cells (Invitrogen). To select positive clones,

plasmid DNA, prepared from cultured individual bacterial clones kanamycin resistant by Plasmid Miniprep Isolation techniques (Biorad), were analyzed by restriction mapping and confirmed by dideoxy Sequencing (Sanger et al.; 1977; Proceedings of the National Academy of Sciences of the USA; 74:5463-5467) of

5   an asymmetric PCR product DNA using pCR II TOPO universal primers (Invitrogen) as sequencing primers.

To construct the final-expression vector encoding the Met-human IL-7 polypeptide, plasmid DNA from a positive clone was digested with restriction endonucleases

10.   BamHI and NdeI and the resulting fragment, r-hIL-7 encoding DNA sequence, was inserted in between BamHI and NdeI restriction sites of a ptac vector.

This expression plasmid was constructed by replacing the T7 promoter by a tac promoter in a classical pET vector derived from pBR322. For this purpose, the tac

15   promoter was first amplified using pMAL-p2X (Biolabs) as a matrix and oligonucleotides :

SEQ ID NO : 9: ptac1
5'ATCG*AGATCT*AATTCTCATGTTTGACAGCTTATCAT3'

20   BglII

SEQ ID NO : 10 : ptac2
5'ATCG*TCTAGA*GCTGTTTCCTGTGTGAAATTGTTATCCG3'
            Xbal

25

The obtained PCR fragment was loaded on an agarose gel to check for its correct size. It was then digested by the BglII and Xbal and inserted into the pET9a (Novagen) hydrolyzed by the same enzymes. The obtained ligation products were transformed into TOP10 (Invitrogen) competent cells and selected on their

kanamycine resistance. The obtained ptac vector was then checked by digestion with several enzymes and by sequencing analysis using oligonucleotides ptac1 and ptac2 as sequencing primers.

5   The ligation products, containing r-hIL-7 fragment, were transformed into TOP10 competent cells. The selection for plasmid-containing cells was on the basis of the antibiotic (kanamycin) resistance marker gene carried on the vector. Plasmid DNA from a positive clone was isolated from cultured cells, selected by restriction mapping and the correct DNA sequence confirmed by sequencing analysis using

10   pET universal primers as sequencing primers (Novagen) as well as a ptac specific primer:

SEQ ID NO : 11 : ptac promoter primer
5'TTCGTGTCGCTCAAGGCGCA3'

15

The *E. Coli* final expression plasmid comprising SEQ ID NO:1, called ptac-hIL-7 (cf. : Figure n°1), was subcloned into *E. coli* JM101 cells (ATCC).

1.2.   Construction of simian IL-7-coding nucleotide sequence:

20

a) Amplification and Sequencing of simian IL-7 cDNA including 5' region

The simian (Macaca Mulatta Rhesus monkey) IL-7 encoding cDNA sequence was obtained from rhesus monkey kidney cDNA (BioChain Inc.) using PCR amplification (Mullis et al.; 1987; Methods in Enzymology; 155:335-350). The basic strategy was

25   to amplify simian IL-7 cDNA by PCR with specific oligonucleotide primers used to amplify human IL-7 cDNA (SEQ ID NO: 5: IL75' and SEQ ID NO: 6: IL73'). A single band was apparent on gel electrophoresis (cf. Figure 6). Asymmetric PCRs and sequencing permit to obtain the simian IL-7 sequence. Sequence homology analysis between human and simian IL-7 DNA and amino acid sequences showed

98.1% and 96.6% of homology-identity for DNA (cf. Figure 4) and amino acid (cf. Figure 5) sequences respectively.

b) Construction of simian IL-7-coding nucleotide sequence

5 Like the human IL-7 sequence, the simian IL-7-encoding DNA sequence presents at position 49 after the "ATG" initiation codon, a second putative "ATG" which could behave as a second initiation codon in E. Coli, since this second "ATG" is preceded by a "pseudo Shine-Dalgarno" sequence (ribosome binding sequence). To avoid the potential production of an amino-terminal truncated form of r-sIL-7, some of the

10 nucleotides of the "SD-like" sequence were mutated (without modifying the resulting encoded r-sIL-7 amino acid sequence), thereby producing an improved methionyl-IL-7-coding-DNA sequence containing one or more preferred codon(s) for expression in E. Coli cells.

15 The amplification of the simian IL-7 encoding DNA sequence lacked of the SD-like sequence was performed by site directed mutagenesis PCR, using oligonucleotide primers: SEQ ID NO: 7: mutIL75' and SEQ ID NO: 6: IL73').

PCR products were analyzed by polyacrylamide or agarose gel electrophoresis in

20 the presence of ethidium bromide and visualization by fluorescence of DNA bands stimulated by UV irradiation. A single major product band of the size corresponding to the IL-7 PCR fragment (cf. Figure 7) was isolated and inserted into the plasmid vector pCR II-TOPO (Invitrogen) using the TA-cloning method. The ligation products were transformed into TOP10 competent cells. To select positive clones,

25 plasmid DNA, prepared from cultured individual bacterial clones kanamycin resistant by Plasmid Miniprep Isolation techniques, were analyzed by restriction mapping and confirmed by dideoxy Sequencing (Sanger et al.; 1977; Proceedings of the National Academy of Sciences of the USA; 74:5463-5467) of an asymmetric

PCR product DNA using pCR II TOPO universal primers (Invitrogen) as sequencing primers yielded about 700 bases containing sIL-7 sequences.

5   Plasmid DNA from a positive clone was digested with restriction endonucleases BamHI and NdeI and the resulting fragment, r-sIL-7 encoding DNA sequence, was inserted into ptac vector, as descibed in example 1.1., which was also digested with *BamH*I and *Nde*I restriction sites. The ligation products were transformed into TOP10 competent cells. The selection for plasmid-containing cells was on the basis of the antibiotic (kanamycin) resistance marker gene carried on the vector. Plasmid

10   DNA from a positive clone was isolated from cultured cells, selected by restriction mapping and confirmed by sequencing analysis using T7 terminator universal primer on one hand and ptac promoter primer on the other hand as sequencing primers.

15   The *E. Coli* final expression plasmid comprising SEQ ID NO:-12, called ptac-sIL7opt (cf. : Figure n°2), was subcloned into *E. coli* JM101 cells.

A2.   Mammalian Expression (BHK Cell Expression, or CHO Cell Expression or HEK-293 Cell Expression):

20

The human IL-7 encoding cDNA sequence was amplified by polymerase chain reaction (PCR) (Mullis et al.; 1987; Methods in Enzymology; 155:, 335-350) from human placenta cDNA (BioChain Inc.), using oligonucleotides as primers in attempts to amplify the portions of the cDNA of interest: the IL-7 cDNA fragments

25   linked at the 5' end to the natural IL-7 peptide signal.

The following oligonucleotides were used, which contain restriction endonuclease recognition sequences and kozak sequence:

- SEQ ID NO: 8: PSIL75'

5'GC*AAGCTT*GCCACCATGTTCCATGTTTCTTTTAGGTATATCTTTGGAC3'

H*ind*III   Kozak sequence

5   - SEQ ID NO: 6: IL73'

5'AGCC*GGATCC*TTATCAGTGTTCTTTAGTGCCCATCA3'

B*am*HI

PCR products were assayed by polyacrylamide or agarose gel electrophoresis in
10   the presence of ethidium bromide and visualization by fluorescence of DNA bands
stimulated by UV irradiation (cf. figure 8). The product band of the size
corresponding to the IL-7 PCR fragment, designated "hPSIL-7 cDNA", was isolated
and inserted into the plasmid vector pCR II-TOPO (Invitrogen) using the TA-cloning
method. The ligation products were transformed into TOP10 competent cells. To
15   select positive clones, plasmid DNA, prepared from cultured individual bacterial
clones ampicilin resistant by Plasmid Miniprep Isolation techniques (Biorad), were
analyzed, by restriction mapping and confirmed by dideoxy sequencing (Sanger et
al.; 1977; Proceedings of the National Academy of Sciences of the USA; 74:5463-
5467) of an asymmetric PCR product DNA using pCR II TOPO universal primers as
20   sequencing primers.

Plasmid DNA from a positive clone was digested with restriction endonucleases
HindIII and BamHI and the resulting fragment, "hPSIL-7 cDNA" fragment, was
inserted into pcDNA3.1(+) vector polylinker (Invitrogen) which had been digested
with   *Bam*HI and *Hind*III restriction sites. This vector includes a gene for the
25   selection and amplification of clones. The ligation products were transformed into
TOP10 competent cells. The selection for plasmid-containing cells was on the basis
of the antibiotic (ampicilin) resistance marker gene carried on the vector. Plasmid
DNA from a positive clone was isolated from cultured cells, selected by restriction

mapping and confirmed by sequencing analysis using pcDNA3.1(+) universal primers as sequencing primers (Invitrogen). The expression system was designed to express an IL-7 protein predicted from the translation of the natural human IL-7 gene sequence. Selection for recombinant vector-containing cells was done on the
5   basis of the antibiotic (Ampicilin for cloning in *E. Coli* and Neomycin for expression in mammalian cells) resistance marker gene carried on the vector.

The mammalian (HEK-293, CHO or BHK) expression vector comprising SEQ ID NO:3, is called pcDNA-hPSIL-7 (cf.: Figure n°3). Expression of human IL-7 in HEK-
10   293 or CHO transfected cells was achieved using the expression vector pcDNA-hPSIL-7. After linearization by Bg*l*II, expression vector, pcDNA-hPSIL-7, was transfected in the mammalian host cells using methods known to those skilled in the art. The selectable marker used to establish stable transformants was G418 (Invitrogen).

15

Example B. Fermentation of E. coli producing recombinant IL-7

Fermentations for the production of recombinant (human or simian) IL-7 were carried out in 80 liters fermentor (New Brunswick) using an *E. coli* JM101 host strain
20   transformed with expression plasmid ptac-hIL-7 or ptac-sIL7opt as described in example A1.

1 L inoculum culture was aseptically transferred into fermentor containing 50 L batch medium NRJ18 (pH7). The culture was grown in batch mode (T 37°C, agitation: 100-500 rpm D.O2: 30%). The production phase of the fermentation was
25   induced by IPTG (200 mg/L) after D.O2 reached to 0% until the OD-600 of the culture was about 40. The fermentor content was collected and cooled for at least 30 min in order to reach temperature under 20°C. The culture media was filtered using a 70 μm filter (PALL R1F-700) to eliminate precipitates and cells were harvested by centrifugation (Beckman J6) at 5000 g for 30 min at 4°C.

Example C. Fermentation of HEK-293 cells producing recombinant hIL-7

The best stable positive clone, as in example A2 was adapted to serum-free
5   suspension culture by several media screenings in order to produce a clone
optimized for productivity and growth in high cell density culture. Cell culture was
performed in a 3 liters bioreactor with a perfusion system. The cells were allowed to
grow to a concentration of 10 millions cells/ml. The reactor was operated at a
continuous perfusion rate of approximately 3 L/day during 10 days. Roughly 30 L of
10   serum-free culture media containing recombinant protein were generated and used
as starting material for the purification of the r-hIL-7.

Example D. Purification of recombinant IL-7 product Expressed in E. Coli

15   The harvested cells, as in example B, were suspended in Tris20mM/EDTA10mM
buffer (pH8) and centrifuged at 16900 g for 45 min at 4°C. After two successive
washing/centrifugation cycle, inclusion bodies fractions were recovered.
This inclusion bodies fraction was diluted in order to obtained a protein
concentration of 5 to 6 mg/ml and solubilized in solubilization buffer (8 M guanidine
20   hydrochloride - 1 mM EDTA - 1% b-mercaptoethanol - 0.5% DMDAP - 10 mM
sodium phosphate - pH8) giving a full reduction, denaturation and solubilization of
the protein. The solution was diluted 1.6 fold in sodium phosphate buffer 6.25 mM
and adjusted to 1.5 M ammonium sulfate, pH7. The final concentration of guanidine
hydrochloride reached 5M.
25   The solubilized inclusion bodies were pre-filtered and then loaded onto an HIC Butyl
650M (Toso Haas) column equilibrated with the loading buffer (6.25 mM sodium
phosphate - 5 M guanidine hydrochloride - 1.7 ammonium sulfate - pH7). After
sample application and washing of the column with the same buffer, elution is
carried out in one step with 100% of elution buffer (6.25 mM sodium phosphate, 5 M

guanidine hydrochloride, pH 7). All the fractions were collected, pooled and adjusted to a O.D.280 = 0.5 by diluting in 6.25 mM sodium phosphate pH 7, 5 M guanidine hydrochloride buffer. During this step, various contaminants, among which DNA, which would lower the recovery of the subsequent refolding step, were

5   eliminated.

IL-7 was allowed to renature by dilution into the refolding buffer: the pooled fractions from HIC was diluted 2.5 fold in 83.3 mM Tris buffer, 0.16 % tween 80, 0.5 M L-arginine, 0.166 mM oxydized gluthatione and 1.6 mM reduced gluthatione pH 8.5. The dilution process was performed on a linear mode, over a 2 hours period, while

10   stirring the receiving buffer. The renaturation step could be performed fixed on a solid support.

Renaturated IL-7 was loaded in two times onto either membrane filtration or a G25 Sephadex (Pharmacia) column equilibrated with elution buffer (20 mM sodium phosphate, 0.2 M L-arginine, pH 7) and eluted in order to allow the proper load of r-

15   IL-7 on the subsequent affinity support.

The protein peak obtained from the G25 step was loaded onto an Heparin Sepharose Fast Flow (Pharmacia) column equilibrated with a loading buffer (20 mM sodium phosphate, 50 mM sodium chloride, pH 7). After sample application and washing of the column with the loading buffer, elution was carried out in two steps.

20   At first, a fixed ratio of 25 % elution buffer (20 mM sodium phosphate, 1 M sodium chloride, pH 7) / loading buffer was applied over 20 column volumes. Then, refolded IL-7 was eluted in one step with 60 % of elution buffer / loading buffer. The strong selectivity of this step conducted to the elution of the correctly refolded r-IL-7 conformer.

25   In order to eliminate most of the residual impurities including endotoxins, the fraction was adjusted to pH 5 and subjected to a Carboxymethyl Ceramic (BioSepra) column. The CMC column was equilibrated with loading buffer (50 mM sodium acetate, pH 5). After sample application and washing of the column with washing buffer (50 mM sodium acetate, sodium chloride 0.2 M, pH 6), elution was

carried out in one step with buffer (50 mM sodium acetate, 0.8 M sodium chloride, pH 6). Finishing steps may also included a G25 Sephadex purification step for desalting followed by a Q Sepharose Fast Flow (QFF Pharmacia) which retained various residual contaminants. R-IL-7 drug substance was recovered pure in the

5   flow through, as showed in figure 9 representing SDS-PAGE analysis: coomassie blue colored and silver stained.

Example E. Purification of recombinant human IL-7 product Expressed in HEK-293 cells

10

Crude cell culture fluid, generated by growth of expression system HEK-293-pcDNA-hPSIL-7 as reported in example C, was treated using a traditional approach or a Streamline ion exchanger mode.

In a Streamline procedure, crude cell culture fluid was transferred directly from the

15   fermentor to the expanded bed of Streamline rProtein A, for example, according to the protocol supplied by the manufacturer (Amersham Biosciences)

In a traditional approach, crude cell culture fluid was clarified used a combination of microfiltration (0.45 μm) ultra/diafiltration to isolate the product.

The protein solution obtained was loaded onto an Heparin Sepharose Fast Flow

20   (Pharmacia) column to purify the product.

Finishing steps may also included a Carboxymethyl Ceramic (BioSepra) purification step for eliminating residual impurities followed by a G25 Sephadex purification step for desalting followed by a Q Sepharose Fast Flow (QFF Pharmacia) which retained various residual contaminants. R-IL-7 drug substance was recovered pure in the

25   flow through of the last purification step.

Example F. Product controls and specifications

F1.   Drug Substance controls and specifications:

CONTROLS	TESTS
IDENTITY	Western Blot Size-exclusion HPLC
PROCESS RELATED IMPURITIES	ECP Dosage Bacterial Endotoxins Dosage LAL test DNA Quantification (Hybridation, quantitative PCR) SDS-PAGE Silver stain
PRODUCT RELATED IMPURITIES	Reverse phase HPLC Ion Exchange HPLC Size-exclusion HPLC Heparin Affinity HPLC (this control warrant the obtention of the correctly refolded Drug Substance)
STERILITY	Microbiology sterility
PURITY	Size-exclusion HPLC
POTENCY	Bioassay
QUANTITY	BCA ELISA Size-exclusion HPLC UV Absorbtion

F2.   Example Lot X r-. hIL-7 Batch Results

TEST	METHODS	SPECIFICATIONS	OBTAINED
CHARACTERS			
Appearance	Visual control	Colorless liquid	comply
pH	PH meter	pH = 6.2 ± 0.1	comply
IDENTIFICATION			
Western blot	Internal Monograph	Main band recognized by the specific anti-hIL-7 antibody at 17.5 kDa	comply
Size-exclusion HPLC	Internal Monograph	Retention time: 23.7 +/- 0.2 mn	comply
TEST			
Purity by SE-HPLC Quantity by SE HPLC	Internal Monograph	Polypeptidic purity ≥ 96% (including dimer)	99 % No Dimer
Purity by RP-HPLC	Internal Monograph	Polypeptidic Purity ≥ 96%	96 % secondary forms

Purity by SDS-PAGE (Silver stained)	Internal Monograph	Polypeptidic Purity ≥ 96% (including dimer)	98 %
Purity by Cation exchange HPLC	BIP Internal Monograph	Polypeptidic Purity ≥ 96%	96 % including secondary forms
Host Proteins by ELISA	BIP Internal Monograph	< 100 ppm	50 ppm
Plasmid DNA by hybridization	BIP Internal Monograph	<100 pg/dose < 10 pg/mg protein	20 pg/dose
Endotoxins	BIP Internal Monograph	<100 EU/dose <10 EU / mg protein	3 EU/mg
Sterility	BIP Internal Monograph	Sterile	comply
ASSAY			
IL-7 content by ELISA assay	BIP Internal Monograph	50%< Standard Potency<150%	90 % of standard
Total protein content by BCA assay	BIP Internal Monograph	Quantification	40 mg/L – 60 mg/L

Characterization and proof of structure of this conformer included an amino acid analysis, a peptide mapping after tryptic digest, the sequence of the amino-terminal part, a mass spectrometry (MALDI TOF) control of the molecular weight, a mass spectrometry control of the disulfide bridges, and protein profiles through: SDS PAGE silver stain, Reverse phase HPLC, Cation exchange HPLC, Size exclusion HPLC.

F3.   Peptide mapping

Peptide sequences was conform with expected sequences of a r-hIL-7 tryptic digest

Mass	Position	Peptide sequence
911.324	1-8	MDCDIEGK
319.161	9-11	DGK
2099.040	12-29	QYESVLMVSIDQLLDSMK
1775.806	30-44	EIGSNCLNNEFNFFK
175.119	45-45	R
800.372	46-52	HICDANK

899.444	53-59	EGMFLFR
317.193	60-62	AAR
147.113	63-63	K
288.203	64-65	LR
535.324	66-69	QFLK
1490.731	70-82	MNSTGDFDLHLLK
1662.873	83-98	VSEGTTILLNCTGQVK
232.140	99-100	GR
1210.679	101-112	KPAALGEAQPTK
719.357	113-118	SLEENK
347.229	119-121	SLK
404.214	122-124	EQK
147.113	125-125	K
965.512	126-133	LNDLCFLK
175.119	134-134	R
743.466	135-140	LLQEIK
651.292	141-145	TCWNK
662.391	146-151	ILMGTK
285.119	152-153	EH

F4.   Mass spectrometry MALDI-TOF : Protein MW

Average mass of protein with one charge (M+H)+. The measurement gave a molecular mass of 17517.6 Da for a theoretical value of 17518.4 Da

F5.   Mass spectrometry MALDI-TOF : Sulfydryl groups

Mass of monoisotopic peptides charged one time (M+H)+.

Calculated mass.	Measured mass.	peptide	sequence	observations
535.32	535.22	66-69	QFLK	
662.39	662.31	146-151	ILMGTK	
719.36	719.29	113-118	SLEENK	
743.47	743.40	135-140	LLQEIK	
899.44	899.44	53-59	EGMFLFR	
1210.68	1210.68	101-112	KPAALGEAQPTK	
1448.64	1448.66	46-52/141-145	HICDANK-TCWNK	Disulfide bridge C48-C142
1490.73	1490.75	70-82	MNSTGDFDLHLLK	
2099.04	2099.04	12-29	QYESVLMVSIDQLLDSMK	
				Disulfide bridge C3-C93
2570.22	2570.23	1-8/83-98	MDCDIEGK-VSEGTTILLNCTGQVK	

2738.30	2738.28	30-44/126-133	EIGSNCLNNEFNFFK-LNDLCFLK	Disulfide bridge C35-C130

Example G. *In vitro* biological activity assay of recombinant IL-7:

Mammalian (human and simian) IL-7 expressed in *E.coli* (Example A1), purified and characterized as in example D, were assayed and compared to murin IL-7 (R&D System) for their ability to cause the proliferation of the IL-7 dependent cell line designated as pre-B cell line PB1 (DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), a cell line derived from bone marrow cells from CBA/C57BL mice.

Also, human and simian r-IL-7 "impure", generated from purification process using partial denaturation step or by chemically-treated purified r-IL-7 in order to generate about 20% of deamidated IL-7 form or dimer form, were assayed.

The PB-1 cell line is absolutely dependent upon exogenous IL-7 for continued growth and viability. The addition of r-IL-7 to theses cultured cell lines stimulates a dose dependent proliferation allowing for a quantitative determination of the levels of r-IL-7 present. The amount of proliferation is measured by "pulsing" each microwell with tritiated thymidine 3H-TdR for 4 hours at 37°C. Dividing pre-B cells will incorporate 3H-Tdr into their DNA. The cells from each well are then harvested onto glass fiber filter discs which trap the DNA. The amount of specifically-bound radioactivity is then measured in a liquid scintillation counter. The number of counts per minute (cpm) for each well is directly proportional to the amount of proliferation by the activated pre-B cells in response to IL-7.

All IL-7 proteins were active on Murine pre-B cell line as indicated in figure 10.

Example H. Assays for detection of antibodies to recombinant IL-7 in serum:

Human recombinant IL-7 is heterologeous for monkey models and may induce neutralizing antibodies against recombinant human IL-7 conformer (as describe above). These antibodies may function as inhibitors *in vivo* and may contribute to the immunosuppressive therapeutic effects of the drug substance. On this basis, anti-IL-7-antibodies were investigated in serum and plasma of animals treated with r-hIL-7 or r-sIL-7 pure (according to the present invention) or r-sIL-7 "impure" as describe in next examples.

Methods used for the detection of antibodies in body fluids may included cytokine bioassay, immunometric assay, radioligand assay and various blotting techniques. The plasmatic quantification of anti-r-IL-7 antibodies were achieved using ELISA technique and the serological detection of anti-r-IL-7 antibodies were realized using Western blot technique. These techniques are known to those skilled in the art.

H1. ELISA procedure:

ELISA Strip Plate was coated with r-IL-7 (human or simian) diluted in Blocking buffer containing tween at 0.75 µg/ml. After overnight incubation at 4°C, the saturation of the plate was performed with Blocking Buffer for 1 h at RT. After removing all solution and washing the plate with Washing Buffer, the plate was ready to use for the assay.

Serial dilutions of preimmune and immune plasma were performed and dispensed to wells. Plate was incubated for 1 h at 37°C. Positive control (biotyniled IL-7 antibody) and negative control (serum of placebo monkey) were tested as well in specific wells. Plate was then washed with Washing Buffer and incubated, at RT for 30 min with IgG monkey-HRP diluted in Blocking Buffer / tween for serum and negative control. Positive control wells were incubated with streptavidine HRP in Blocking Buffer / tween. After removing the solution, the plate was washed with

Washing Buffer and incubated, at RT for up to 15 min with OPD Substrate solution followed by addition of sulfuric acid in each well to stop the reaction.

The absorbance was then read at 492 nm.

5   ## H2.   Western blot procedure

Recombinant (human or simian) IL-7 was transferred onto nitrocellulose or PVDF membrane. Then the membrane was incubated with the sera of treated animals (dilution of 1/100 or 1/200) or with anti-IL-7 antibody (Anti-human IL-7 antibody: AB 207 NA, R&D System) for the positive control. After removing the solution, the
10   membrane was washed and incubated with the secondary antibody: Anti-monkey IgG alkaline phosphatase conjugate (SIGMA A1929) or Anti-goat IgG alkaline phosphatase conjugate (SIGMA A4187) for the positive control. Then, the revelation was performed using BCIP/NBT (SIGMA).

15   ## Example I. In vivo effects of r-IL-7 on CD4 T cell count in normal non human primate model

Recombinant IL-7 (human and simian) expressed in E.coli (Example A1), purified and characterized as in example D, and r-sIL-7 "impure" (as defined in example G)
20   were tested for in vivo biological activity in normal primates. This primate model was the only possible model to test the long term activity of the drug substance and the immunogenicity of the same drug substance contaminated with molecular variants and/or drug product related impurities. To test the same effect in rodents would be inconclusive: first because rodent sequences show an important deletion in
25   comparison to primate sequences, second because in rodent, IL-7 has a strong lymphopoietic effect on B cells, susceptible to mask the T cell lymphopoietic effect or to prevent from a clear discriminative analysis of this effect. For this reason, in order to prove the effect in primates, while avoiding the unethical testing of an

immunogenic preparation in human, we had to clone and express and purify simian IL-7 and test it in monkey.

Young Macaca rhesus monkeys were studied in four groups (1 to 4), each group contained 3 animals.

5 Monkeys of:

- group 1 received placebo (IL-7 diluent)
- group 2 received r-hIL-7 (according to the present invention) at a dose of 150 μg/kg administered once daily by subcutaneous injection for 4 weeks
- group 3 received r-sIL-7 (according to the present invention) at a dose of
10   150 μg/kg administered once daily by subcutaneous injection for 4 weeks
- group 4 received single daily subcutaneous administration of r-sIL-7 "impure" at a dose level of 150 μg/kg for 4 weeks.

All animals were studied during 6 weeks: 4 weeks of treatment and 2 weeks for reversibility period.

15 Peripheral blood specimens were collected from animals under ketamine restraint before and after IL-7 treatment (on days 0 (pretreatment), 7, 14, 21, 28, 35 and 42). CD4 cellularity was examined on a FACScan cell analysis system (Becton Dickinson). Serum samples were examined for the presence of antibodies by western blot analysis and confirmed by ELISA analysis, as describe in example H.

20 Blood CD4 T cell count is indicated in figure 11: represented as a median of blood CD4 T cell count for each group.

All animals survived the protocol and tolerated IL-7 administration without adverse reactions to IL-7 therapy.

The various IL-7 administered had a moderate effect on blood CD4 T cell count of
25 normal monkeys: peripheral blood CD4 T cell count increased 1.4 to 2.1X from day 14 until day 35 and remain above pretreatment values at day 42 in group 3 (treated with r-sIL-7) compared to placebo group and CD4 cellularity began to increase and then decreased from week 2 until week 6 for groups 2 (treated with r-hIL-7) and 4 (treated with r-sIL-7 "impure" preparation).

Anti-IL-7 antibodies were detected in the sera of these two groups of monkeys (2 and 4), during the second week after receiving r-hIL-7 and r-sIL-7 "impure" respectively, by subcutaneous injection (cf. figure 12). In these normal monkeys, the repeated injection of heterologous or impure IL-7 drug substance did trigger the appearance of anti-IL-7 antibodies and simultaneously this produced a strong decrease of the IL-7 lymphopoietic effect.

Example J. *In vivo* activity of r-sIL-7 in irradiated monkeys

Recombinant simian IL-7 expressed in *E.coli* (Example A1), purified and characterized as in example D, and r-sIL-7 "impure" (as defined in example G) were tested for mid or long term *in vivo* biological activity in immunodepressed primates.

Young rhesus monkeys were studied in three groups (1 to 3), each group contained 3 animals.

All monkeys underwent total body irradiation (TBI) (6.1 Gy).

Monkeys received following regimens:

- group 1 received placebo (IL-7 diluent)
- group 2 received r-sIL-7 (according to the present invention) at a dose of 70 µg/kg administered once daily by subcutaneous injection for 4 weeks starting on day 14 post-irradiation.
- group 3 received single daily subcutaneous administration of r-sIL-7 "impure" at a dose level of 70 µg/kg for 4 weeks starting on day 14 post-irradiation.

All animals were studied during 10 weeks: 2 weeks for post-irradiation hematological recovery period, 4 weeks for IL-7-treatment period and 4 weeks of follow up period.

Blood specimens, for CD4 T cell count, were collected from the animals under ketamine restraint on days 0, 7 and 14 pretreatment, 21, 28, 35 and 42 of treatment, 49, 56, 63 and 70 post-irradiation.

CD4 cellularity was examined on a FACScan cell analysis system (Becton

5   Dickinson) and serum samples were examined for the presence of antibodies using ELISA and western blot techniques as describe in example H.

All animals survived the protocol and presented no adverse reactions to the IL-7 treatment.

As indicated in figure 13, IL-7 administration increased significantly (4.2-fold) the

10 CD4 T cell in the peripheral blood of immunocompromised monkeys of groups 2 and 3 compared to untreated control group. The group 3 treated with subcutaneous administration of r-sIL-7 "impure" display a diminution of the *in vivo* biological effect of IL-7 starting after 3 to 4 weeks of IL-7 treatment.

Anti-IL-7 antibodies were measured in the serum of these animals every week after

15 beginning of IL-7 treatment. Anti-IL-7 antibodies were not detected up to week 10 (8 weeks post-treatment) in group 2 treated with r-sIL-7 (according to the present invention). In contrary, group 3, treated with an impure preparation showed detectable antibodies as soon as week 6 (4 weeks after beginning of IL-7 treatment). At week 10, antibodies were clearly present and exogenous IL-7 had no

20 lymphopoietic effect.

CLAIMS

5   1. A Purified or isolated IL-7 conformer, wherein said conformer comprises the following three disulfide bridges: Cys: 1-4 (Cys2- Cys92); 2-5 (Cys34- Cys129) and 3-6 (Cys47- Cys141).

2. IL-7 conformer according to claim 1, wherein said IL-7 conformer is a
10   recombinant human IL-7 conformer.

3. IL-7 conformer according to claim 2, wherein said IL-7 conformer comprises the amino acid sequence SEQ ID NO: 2 or 4.

15   4. IL-7 conformer according to claim 1, wherein said IL-7 conformer is a recombinant simian IL-7 conformer.

5. IL-7 conformer according to claim 4, wherein said IL-7 conformer comprises the amino acid sequence SEQ ID NO: 12.

20
6. IL-7 conformer according to anyone of claims 1 to 5, wherein said IL-7 conformer is not glycosylated.

7. IL-7 conformer according to anyone of claims 1 to 5, wherein said IL-7
25   conformer is glycosylated.

8. IL-7 conformer according to anyone of claims 1 to 7, wherein said IL-7 conformer is associated to the hepatocyte growth factor as a heterodimer.

9. IL-7 conformer according to anyone of claims 1 to 7, wherein said IL-7 conformer is functionally attached to a Fc portion of an IgG heavy chain through a peptide hinge region, said IgG being a human IgG1 or IgG4.

10. An IL-7 drug substance comprising, as the desired product, an IL-7 conformer according to anyone of claims 1 to 9, said drug substance being substantially free of IL-7 molecular variants or product related impurities.

11. An IL-7 drug substance comprising, as the desired product, an IL-7 conformer according to anyone of claims 1 to 7, said drug substance being substantially free of IL-7 molecular variants or product related impurities, wherein the total amount by weight of IL-7 in said drug substance is at least 95% by weight, preferably at least 98% by weight, more preferably at least 99.5% by weight.

12. A pharmaceutical composition comprising an effective amount of a drug substance according to claim 10 or 11 and one or more pharmaceutically compatible carriers.

13. Pharmaceutical composition according to claim 12, wherein the pharmaceutically compatible carrier is selected from sucrose, trehalose and an amino acid.

14. Pharmaceutical composition according to claim 13, wherein the pharmaceutically compatible carrier is contained in an appropriate buffer to form an isotonic solution.

15. Pharmaceutical composition according to any one of claims 12 to 14, wherein said appropriate buffer has a pH range comprised between 5 to 7.5, preferably 6 to 7, even more preferably of 6.5.

16. A pharmaceutical composition according to claim 15, wherein said appropriate buffer is an organic salt selected from a sodium citrate buffer and an ammonium acetate buffer.

17. A pharmaceutical composition according to claim 12, wherein said composition is a lyophilized form.

18. A pharmaceutical composition according to anyone of claims 12 to 17, further comprising an immuno-stimulating agent selected from a hematopoietic cell growth factor, a cytokine, an antigen and an adjuvant, or a combination thereof, for combined, separate or sequential use.

19. A pharmaceutical composition according to claim 18, wherein said hematopoietic cell growth factor is selected from the Stem Cell Factor (SCF), particularly the soluble form of the SCF, G-CSF, GM-CSF, Flt-3 ligand, IL-15 and IL-2.

20. A pharmaceutical composition according to claim 18, wherein the cytokine is selected from γ interferon, IL-2, IL-12, RANTES, B7-1, MIP-2 and MIP-1α.

21. A pharmaceutical composition according to anyone of claims 18 to 20, wherein said antigen is selected from a synthetic or natural peptide, a recombinant protein, a killed, inactivated or attenuated pathogen product, a lipid, a portion thereof and a combination thereof.

22. A pharmaceutical composition according to claim 21, wherein said antigen is selected from antigens derived from HIV, Varicella Zoster virus, Influenza virus, Epstein Barr virus, type I or 2 Herpes Simplex virus, human cytomegalovirus, Dengue virus, Hepatite A, B, C or E virus, Syncytium respiratory virus, human papilloma virus, mycobacterium tuberculosis, Toxoplasma and Chlamydia.

23. A pharmaceutical composition according to anyone of claims 18 to 22, wherein said adjuvant is selected from any substance, mixture, solute or composition facilitating or increasing the immunogenicity of an antigen and able to induce a Th1-type immune response, such as CpG, QS21 and monophosphoryl lipid A.

24. Pharmaceutical composition according to anyone of claims 12 to 23, for administration to a human patient for prophylactic or therapeutic stimulation of B or T lymphocyte development and proliferation, or for enhancement of global or specific immuno-reconstitution, or for enhancement of humoral or cellular immune response.

25. A pharmaceutical composition according to anyone of claims 12 to 23, to prevent or reduce opportunistic infections in immunodeficient patients.·

26. A pharmaceutical composition according to anyone of claims 12 to 23, to prolong lymphopoiesis stimulation and/or to produce specific immune response and/or to broaden the repertoire of a specific immune response in human patients.

27. A pharmaceutical composition according to claim 24, 25 or 26, wherein human patients are immunodeficient patients, cancer patients, patients

undergoing grafts, patients infected with a virus or a parasite, elderly patients or any patients having low CD4 count.

28. A pharmaceutical composition according to anyone of claims 12 to 27, wherein the effective amount of the drug substance is comprised between about 3 to 300 µg/kg/day, preferably between 10 to 100 µg/kg/day, and in particular administered from once daily down to twice weekly.

29. A nucleic acid molecule encoding an IL-7 polypeptide, wherein said nucleic acid molecule comprises an altered Shine-Dalgarno-like sequence.

30. A nucleic acid molecule comprising a sequence selected from SEQ ID Nos: 1, 3 and 12.

31. A vector comprising a nucleic acid according to claim 29 or 30.

32. A recombinant host cell comprising a nucleic acid according to claim 29 or 30 or a vector according to claim 31.

33. A recombinant host cell according to claim 32, wherein said recombinant host cell is a human cell or a bacterial cell.

34. A recombinant host cell according to claim 33, which is *Escherichia coli* or *Bacillus Brevis*.

35. A recombinant host cell according to claim 33, which is a Chinese Hamster Ovary (CHO) or HEK-293 cell line.

36. An antibody specifically immunoreactive with an IL-7 conformer as defined in anyone of claims 1 to 7.

37. A method of producing an IL-7 drug substance as defined in anyone of claims 10 and 11, the method comprising:

a) providing a sample comprising IL-7 polypeptides,

b) purifying an IL-7 conformer which comprises the following three disulfide bridges: Cys: 1-4 (Cys2- Cys92); 2-5 (Cys34- Cys129) and 3-6 (Cys47-Cys141) to produce an IL-7 drug substance, and

c) optionally, measuring or quantifying, in the drug substance, said particular IL-7 conformer.

38. The method of claim 37, wherein said sample is obtained from recombinant prokaryotic or eukaryotic host cells producing IL-7 polypeptides.

39. The method of claim 38, wherein said sample is (or derives from) a culture of prokaryotic host cells encoding an IL-7 polypeptide and further wherein the method further comprises, prior to step b):

i) treating said sample to cause a complete denaturation of said IL-7 polypeptides,

ii) optionally purifying the denatured polypeptide obtained in step i) and

iii) refolding the polypeptides.

40. The method of claim 39, wherein step i) comprises the dissolution of inclusion bodies in a denaturant buffer.

41. The method of claim 39 or 40, wherein step ii) is performed by hydrophobic chromatography, ion-exchange or inverse phase chromatography.

42. The method of claim 41, wherein said hydrophobic chromatography is implemented using HIC butyl.

43. The method of anyone of claims 39 to 42, wherein step ii) is carried out at a pH comprised between 6 and 9, preferably between 7 and 8,5 inclusive.

44. The method of anyone of claims 39 to 43, wherein said purification step b comprises the performance of an affinity chromatography.

45. The method of claim 44, wherein said affinity chromatography is performed on a column of sulfated polysaccharides.

46. The method of claim 45, wherein the sulfated polysaccharide is dextran sulfate or heparin.

47. The method of any one of claims 37 to 46, wherein the IL-7 conformer is characterized in the drug substance by Mass spectrometry, infra-red spectroscopy, NMR, by determining circular dichroïsm, by measuring the affinity toward a specific monoclonal antibody raised against said IL-7 conformer, or heparin affinity chromatography, and measured or quantified by ELISA, bioassay or the affinity of said IL-7 conformer for IL-7 receptor and any method of protein quantification if applied to the isolated conformer.

48. A method of controlling an IL-7-containing preparation, comprising determining the presence and/or relative quantity, in said preparation, of an IL-7 conformer as defined in any one of claims 1 to 9.

49. A method of producing an IL-7 drug substance or pharmaceutical composition, said method comprising (i) culturing a recombinant host cell

encoding an IL-7 polypeptide, (ii) isolating said recombinant polypeptide to produce an IL-7 drug substance and (iii) optionally, conditioning said IL-7 drug substance to produce a pharmaceutical composition suitable for therapeutic or vaccine use, said method further comprising a step of identifying, characterizing or measuring, in said drug substance or pharmaceutical composition, the quantity and/or quality of an IL-7 conformer as defined in any one of claims 1 to 9 and, more preferably, a step of selecting the drug substance or pharmaceutical composition which comprises, as the active ingredient, more than about 95%, preferably 98% of said IL-7 conformer.

50. The method of claim 49, wherein the quantity and/or quality of said IL-7 conformer is determined by mass spectrometry-related methods, with or without tryptic digest, circular dichroism, NMR, specific monoclonal antibody analysis for disulfide bridges and/or conformation characterization.

51. Use of an IL-7 drug substance obtained by a method according to anyone of claims 37 to 47, for the manufacture of a pharmaceutical composition to induce a prolonged lymphopoiesis stimulation and/or to amplify an immune response.

52. Use of an IL-7 drug substance obtained by a method according to anyone of claims 37 to 47, for the manufacture of a pharmaceutical composition to prevent or treat a disease associated with an immunodeficiency.

PATENT APPLICATION

5

ABSTRACT

10

The present invention relates, generally, to the fields of immunology and molecular biology. The invention discloses, more particularly, new and improved interleukin-7 drug substances, corresponding specific immunoreactive antibodies, as well as compositions comprising the same, their preparation and uses. The invention also

15 discloses methods to characterize the impurity profile of a r-hIL-7 drug substance used for therapeutic purpose, as well as optimized nucleotide sequences encoding mammalian IL-7, recombinant expression vectors and methods for preparing and purifying said polypeptides. The present invention stems from the unexpected discovery that the long term activity of recombinant IL-7 is mostly expressed by a

20 specific conformer and that other conformers, potential product-related substances, product-related impurities, and process-related impurities, which would normally be included in the specification of the drug substance and/or drug product, although bioactive, should be strictly minimized because they are able to trigger an immune reaction against the desired IL-7 molecule.
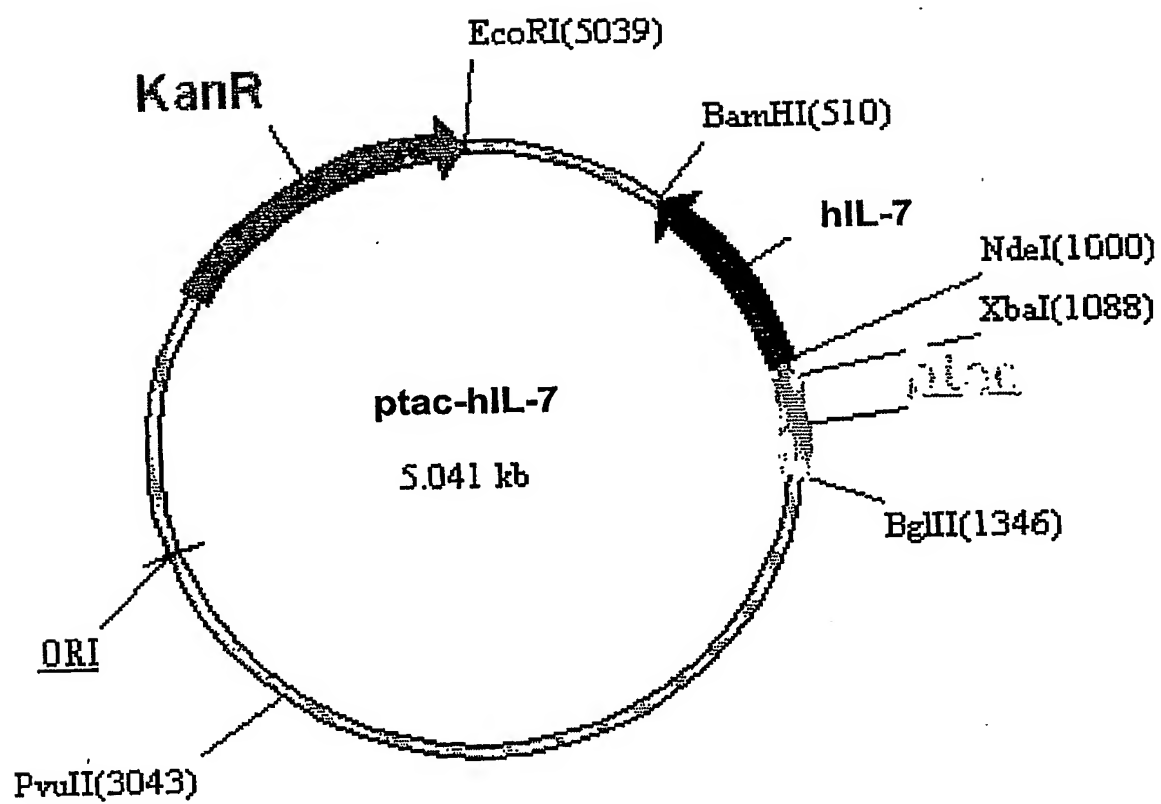
Figure 1
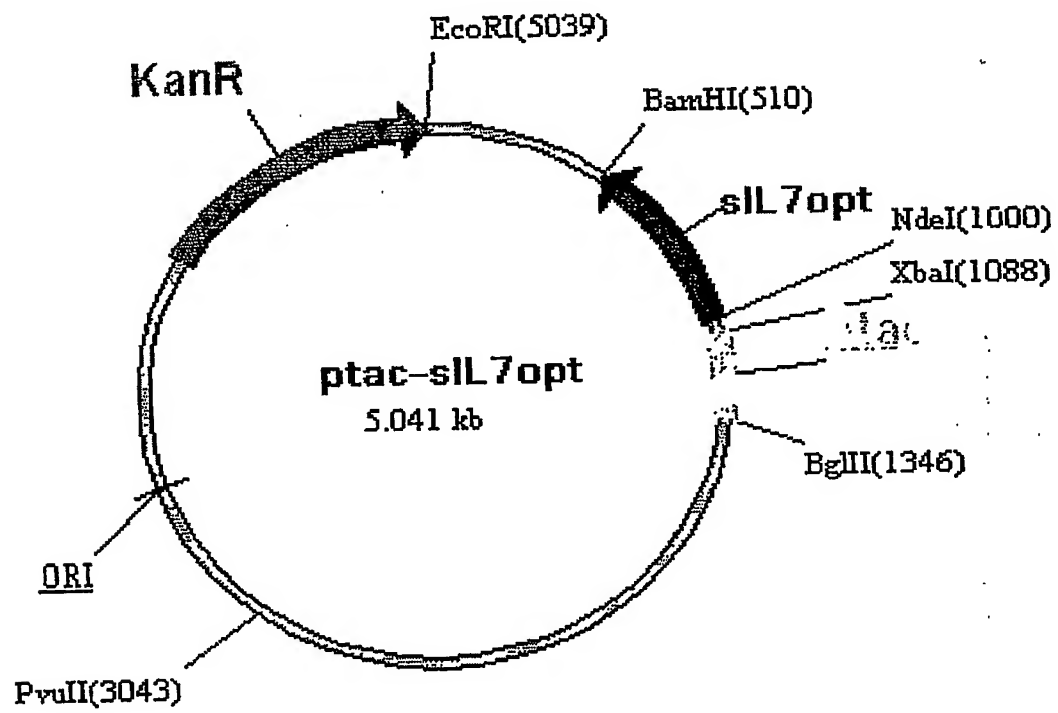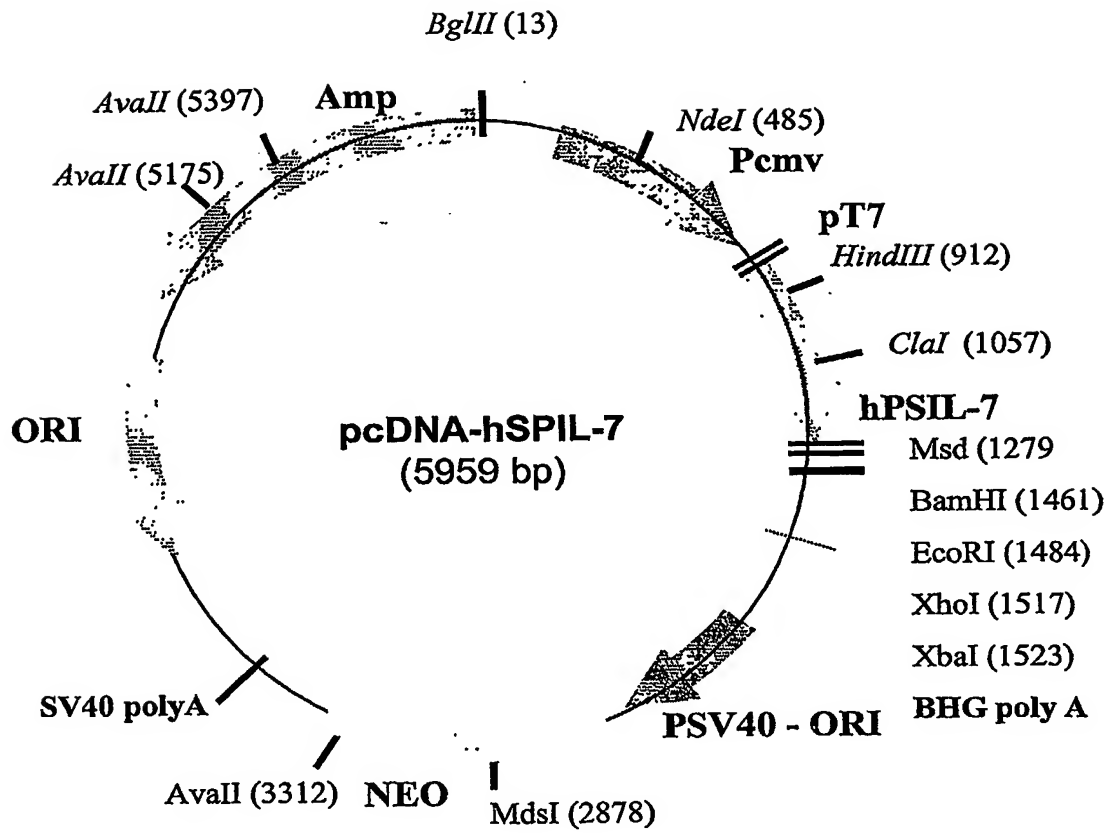
Figure 2

Figure 3

Lalign output for human IL7 vs. monkey IL7 cDNA

98.1% identity in 534 nt overlap; score: 2580 E(10,000): 3.3e-207

```
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human ATGTTCCATGTTTCTTTTAGGTATATCTTTGGACTTCCTCCCCTGATCCTTGTTCTGTTG
monkey ATGTTCCATGTTTCTTTTAGGTATATCTTTGGACTTCCTCCCCTGATCCTTGTTCTGTTG

                70        80        90        100       110       120
human CCAGTAGCATCATCTGATTGTGATATTGAAGGTAAAGATGGCAAACAATATGAGAGTGTT
monkey CCAGTAGCATCATCTGATTGTGATATTGAAGGTAAAGATGGCAAACAATATGAGAGTGTT

                130       140       150       160       170       180
human CTAATGGTCAGCATCGATCAATTATTGGACAGCATGAAAGAAATTGGTAGCAATTGCCTG
monkey CTAATGGTCAGCATCGATCAATTATTGGACAGCATGAAAGAAATTGGTAGCAATTGCCTG

                190       200       210       220       230       240
human AATAATGAATTTAACTTTTTTAAAAGACATATCTGTGATGCTAATAAGGAAGGTATGTTT
monkey AATAATGAATTTAACTTTTTTAAAAGACATCTA TGTGATGATAATAAGGAAGGTATGTTT

                250       260       270       280       290       300
human TTATTCCGTGCTGCTCGCAAGTTGAGGCAATTTCTTAAAATGAATAGCACTGGTGATTTT
monkey TTATTCCGTGCTGCTCGCAAGTTGAGGCAATTTCTTAAAATGAATAGCACTGGTGATTTT

                310       320       330       340       350       360
human GATCTCCACTTATTAAAAGTTTCAGAAGGCACAACAATACTGTTGAACTGCACTGGCCAG
monkey GATCTCCACTTATTAAAAGTTTCAGAAGGCACAACAATACTGTTGAACTGCACCGGCAAG

                370       380       390       400       410       420
human GTTAAAGGAAGAAAACCAGCTGCCCTGGGTGAAGCCCAACCAACAAAGAGTTTGGAAGAA
monkey GTTAAAGGAAGAAAACCAGCTGCCCTGGGTGAACCCCAACCAACAAAGAGTTTGGAAGAA

                430       440       450       460       470       480
human AATAAATCTTTAAAGGAACAGAAAAAACTGAATGACTTGTGTTTCCTAAAGAGACTATTA
monkey AATAAATCTTTAAAGGAACAGAAAAAACTGAATGACTCATGTTTCCTAAAGAGACTACTA

                490       500       510       520       530
human CAAGAGATAAAAACTTGTTGGAATAAAATTTTGATGGGCACTAAAGAACACTGA
monkey CAAA AGATAAAAACTTGTTGGAATAAAATTTTGATGGGCACTAAAGAACACTGA
```

Figure 4

Comparison of :

(A) /tmp/www-sib/lalign.17549.1.seq sp|P13232|
IL7_HUMAN (IL7)INTERLEUKIN-7 PRECURSOR   - 177 AA
(B) /tmp/www-sib/lalign.17549.2.
SEQUENCE MONKEY IL7 PRECURSOR   - 177 AA

96.6% identity in 177 aa overlap; score: 1144 E(10,000): 1.3e-95

```
                 10         20         30         40         50         60
human  MFHVSFRYIFGLPPLILVLLPVASSDCDIEGKDGKQYESVLMVSIDQLLDSMKEIGSNCL
monkey MFHVSFRYIFGLPPLILVLLPVASSDCDIEGKDGKQYESVLMVSIDQLLDSMKEIGSNCL


                 70         80         90        100        110        120
human  NNEFNFFKRHICDANKEGMFLFRAARKLRQFLKMNSTGDFDLHLLKVSEGTTILLNCTGQ
monkey NNEFNFFKRHLCDDNKEGMFLFRAARKLRQFLKMNSTGDFDLHLLKVSEGTTILLNCTGK


                130        140        150        160        170
human  VKGRKPAALGEAQPTKSLEENKSLKEQKKLNDLCFLKRLLQEIKTCWNKILMGTKEH
monkey VKGRKPAALGEPQPTKSLEENKSLKEQKKLNDSCFLKRLLQKIKTCWNKILMGTKEH
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Figure 5

Simian IL-7 PCR products

Figure 6

Optimized simian IL-7
PCR products
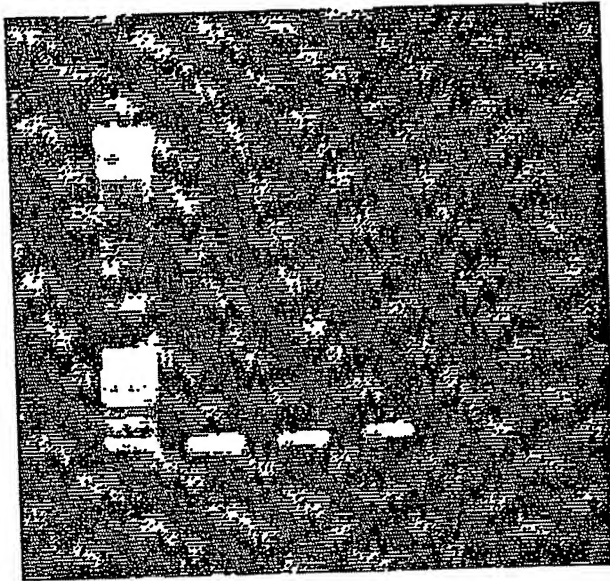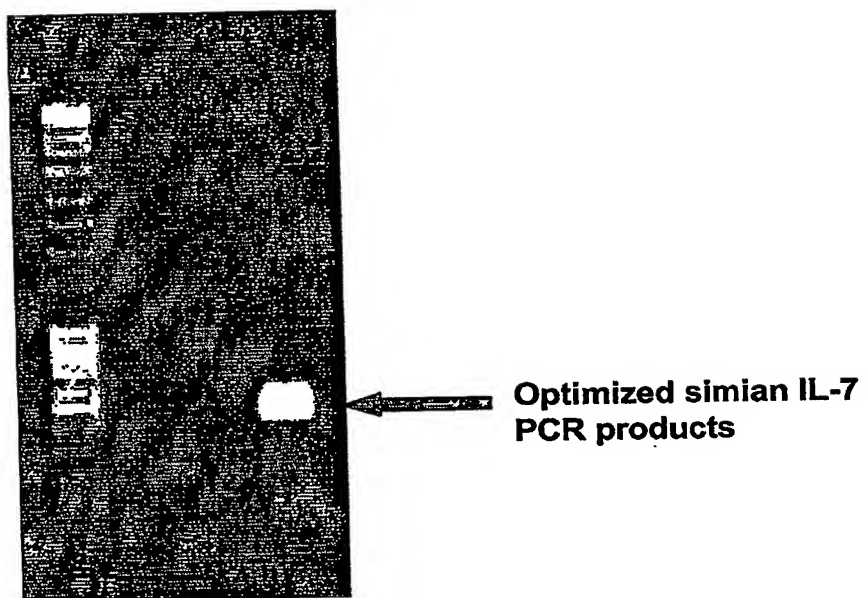
Figure 7

Human PS-IL-7
PCR product

Figure 8

SDS-PAGE Coomassie Blue staining 15%

1	2	MW	1*	2*

kDa
201-
130
94
48.6

36.4

29.8

20.6

1 : purified hIL7 batch A (1µg)

2 : purified hIL7 batch B (1µg)

1* et 2*: reduction condition

MW: BioRad # 161-0318
6.6/20.6/29.8/36.4/48.4/94/130/201

SDS-PAGE Silver staining 15%

1	2	MW	1*	2*

kDa
201-
130
94
48.6

36.4

29.8

20.6

1 : purified hIL7 batch A (1µg)

2 : purified hIL7 batch B (1µg)

1* et 2*: reduction condition

MW: BioRad # 161-0318
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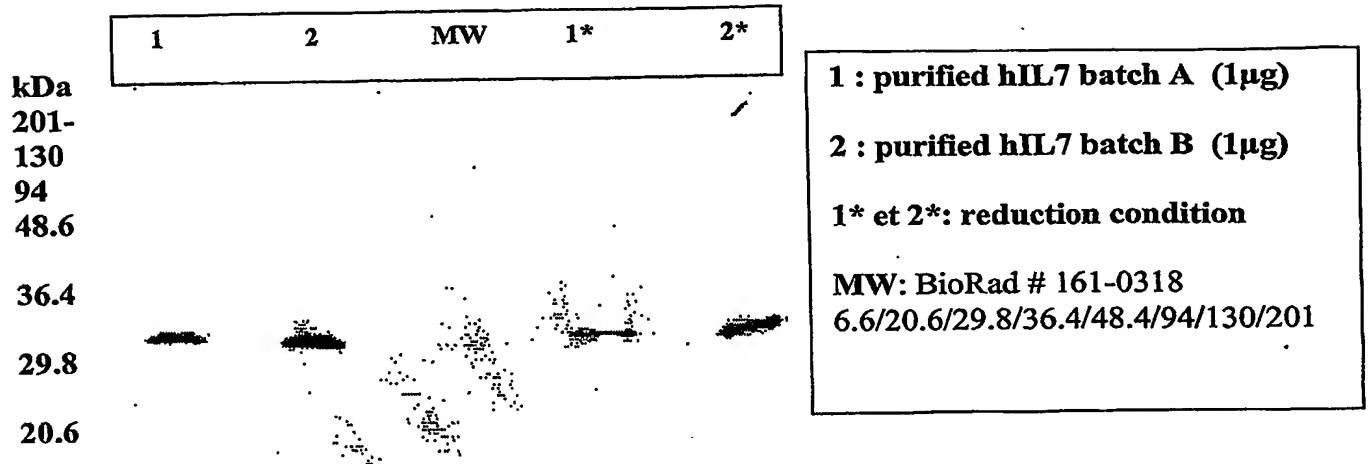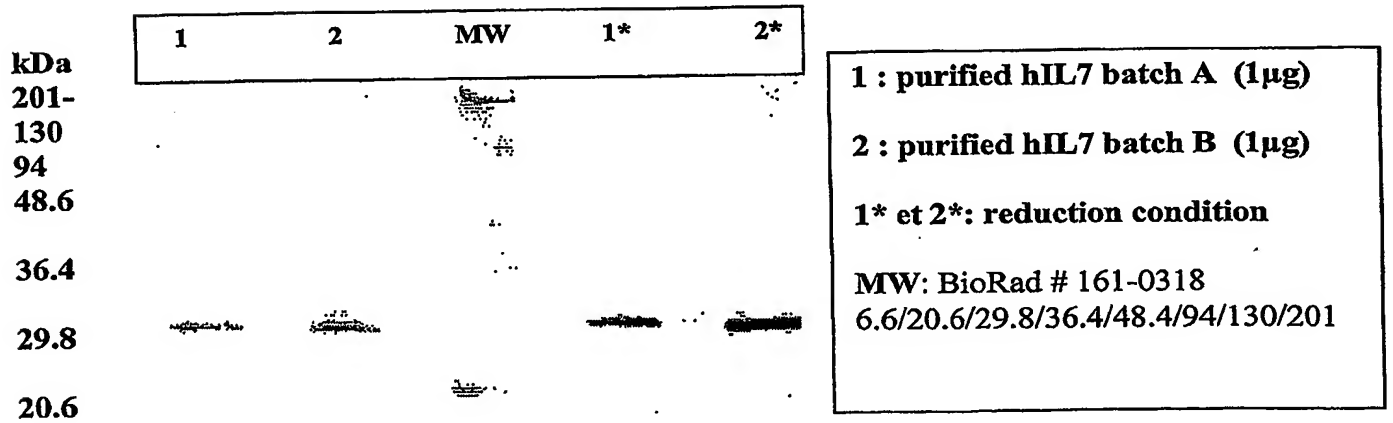
Figure 9

Thymidine Assay with the PB-1 Cell Line
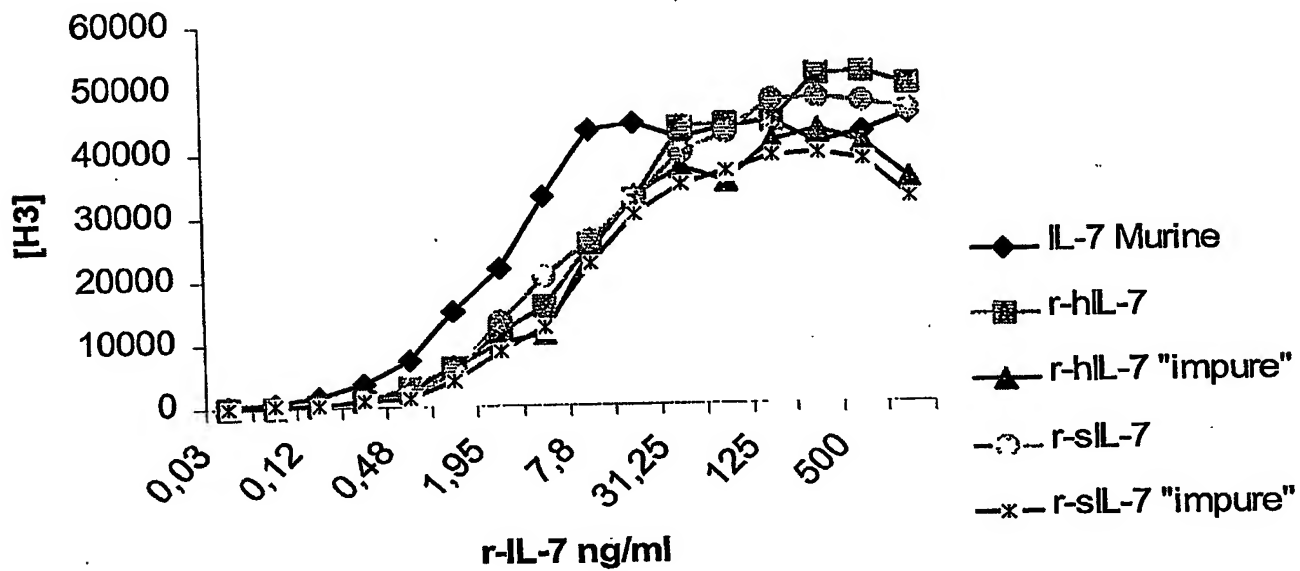
Figure 10

In vivo effects of r-IL-7 on CD4 T cell count in normal non human primates model

r-IL-7 Treatment

— median_placebo
— median_r-sIL-7
— median_r-sIL-7 "impure"
— median_r-hIL-7

CD4/mm3
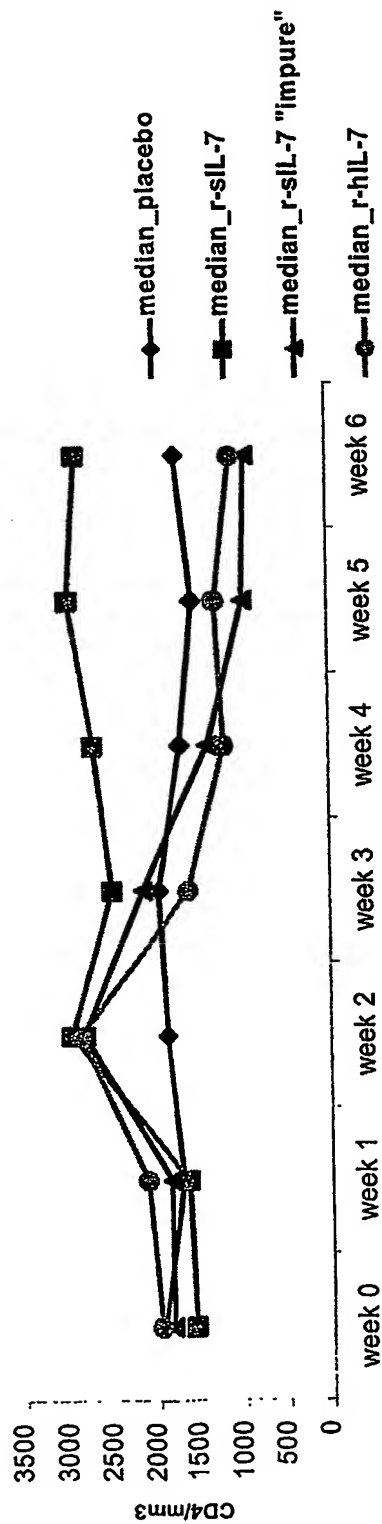
Figure 11

Western Blot analysis of IL-7-treated monkeys sera for the detection of anti-IL-7 antibodies
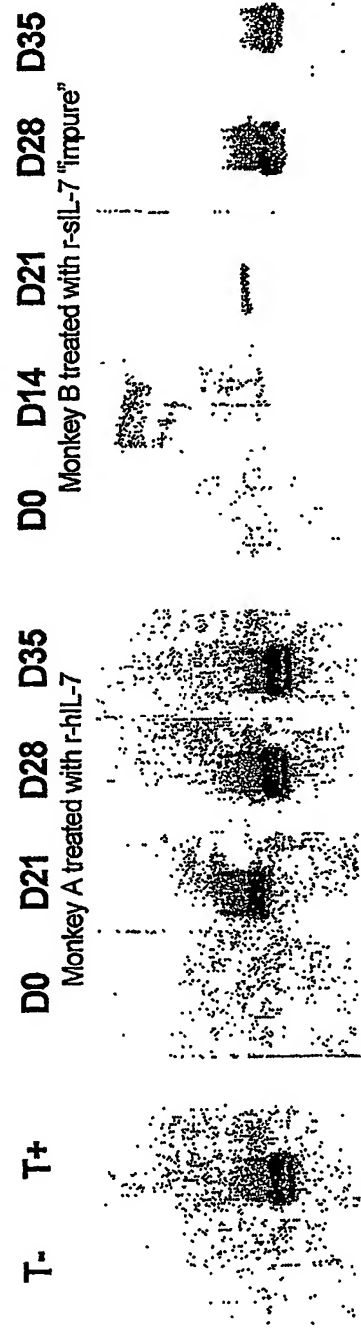
* Monkeys treated with r-sIL-7 remain negative up to day 35 (data not shown)

Figure 12

In vivo activity of r-sIL-7 on CD4 T cell count in irradiated monkey model
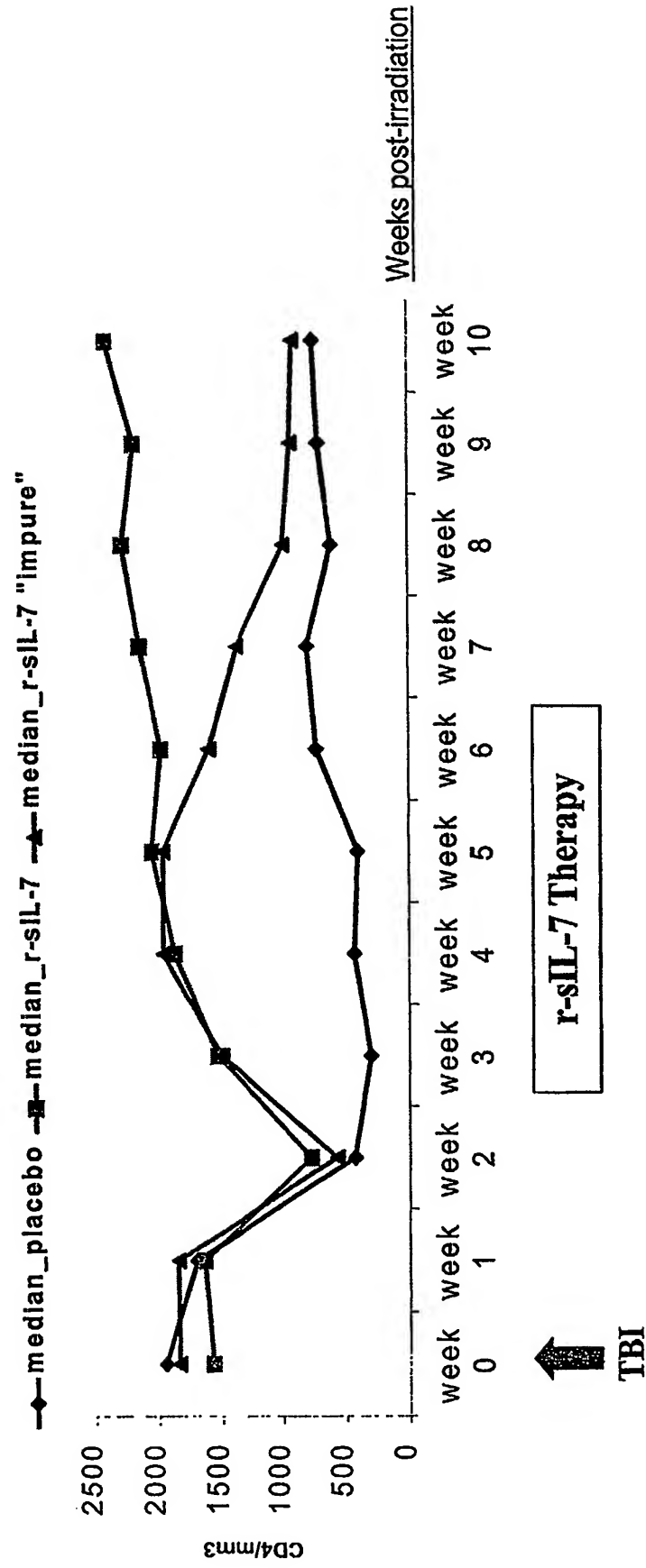
Figure 13

<110> BIOTECH INFLECTION POINT

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ctg atg gtc agc atc gat caa tta ttg gac agc atg aaa gaa att ggt      96
Leu Met Val Ser Ile Asp Gln Leu Leu Asp Ser Met Lys Glu Ile Gly
               20                  25                  30

agc aat tgc ctg aat aat gaa ttt aac ttt ttt aaa aga cat atc tgt     144
Ser Asn Cys Leu Asn Asn Glu Phe Asn Phe Phe Lys Arg His Ile Cys
           35                  40                  45

gat gct aat aag gaa ggt atg ttt tta ttc cgt gct gct cgc aag ttg     192
Asp Ala Asn Lys Glu Gly Met Phe Leu Phe Arg Ala Ala Arg Lys Leu
       50                  55                  60

agg caa ttt ctt aaa atg aat agc act ggt gat ttt gat ctc cac tta     240
Arg Gln Phe Leu Lys Met Asn Ser Thr Gly Asp Phe Asp Leu His Leu
   65                  70                  75                  80
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tta aaa gtt tca gaa ggc aca aca ata ctg ttg aac tgc act ggc cag    288
Leu Lys Val Ser Glu Gly Thr Thr Ile Leu Leu Asn Cys Thr Gly Gln
                85                      90                  95

gtt aaa gga aga aaa cca gct gcc ctg ggt gaa gcc caa cca aca aag    336
Val Lys Gly Arg Lys Pro Ala Ala Leu Gly Glu Ala Gln Pro Thr Lys
                100                 105                 110

agt ttg gaa gaa aat aaa tct tta aag gaa cag aaa aaa ctg aat gac    384
Ser Leu Glu Glu Asn Lys Ser Leu Lys Glu Gln Lys Lys Leu Asn Asp
            115                 120                 125

ttg tgt ttc cta aag aga cta tta caa gag ata aaa act tgt tgg aat    432
Leu Cys Phe Leu Lys Arg Leu Leu Gln Glu Ile Lys Thr Cys Trp Asn
        130             135             140

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                35                  40                  45

Asp Ala Asn Lys Glu Gly Met Phe Leu Phe Arg Ala Ala Arg Lys Leu
    50                  55                  60

Arg Gln Phe Leu Lys Met Asn Ser Thr Gly Asp Phe Asp Leu His Leu
65                  70                  75                  80

Leu Lys Val Ser Glu Gly Thr Thr Ile Leu Leu Asn Cys Thr Gly Gln
                85                  90                  95

Val Lys Gly Arg Lys Pro Ala Ala Leu Gly Glu Ala Gln Pro Thr Lys
                100                 105                 110
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2

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        115                 120                 125

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  1           5                   10                  15

ctt gtt ctg ttg cca gta gca tca tct gat tgt gat att gaa ggt aaa    96
Leu Val Leu Leu Pro Val Ala Ser Ser Asp Cys Asp Ile Glu Gly Lys
                20                  25                  30

gat ggc aaa caa tac gag tcc gtt ctg atg gtc agc atc gat caa tta   144
Asp Gly Lys Gln Tyr Glu Ser Val Leu Met Val Ser Ile Asp Gln Leu
            35                  40                  45

ttg gac agc atg aaa gaa att ggt agc aat tgc ctg aat aat gaa ttt   192
Leu Asp Ser Met Lys Glu Ile Gly Ser Asn Cys Leu Asn Asn Glu Phe
        50                  55                  60

aac ttt ttt aaa aga cat atc tgt gat gct aat aag gaa ggt atg ttt   240
Asn Phe Phe Lys Arg His Ile Cys Asp Ala Asn Lys Glu Gly Met Phe
 65                  70                  75                  80

tta ttc cgt gct gct cgc aag ttg agg caa ttt ctt aaa atg aat agc   288
Leu Phe Arg Ala Ala Arg Lys Leu Arg Gln Phe Leu Lys Met Asn Ser
                85                  90                  95
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3

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Thr Gly Asp Phe Asp Leu His Leu Leu Lys Val Ser Glu Gly Thr Thr
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ata ctg ttg aac tgc act ggc cag gtt aaa gga aga aaa cca gct gcc    384
Ile Leu Leu Asn Cys Thr Gly Gln Val Lys Gly Arg Lys Pro Ala Ala
        115             120             125

ctg ggt gaa gcc caa cca aca aag agt ttg gaa gaa aat aaa tct tta    432
Leu Gly Glu Ala Gln Pro Thr Lys Ser Leu Glu Glu Asn Lys Ser Leu
        130             135             140

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Lys Glu Gln Lys Lys Leu Asn Asp Leu Cys Phe Leu Lys Arg Leu Leu
145             150             155             160

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cac                                                                531
His


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            20              25              30

Asp Gly Lys Gln Tyr Glu Ser Val Leu Met Val Ser Ile Asp Gln Leu
        35              40              45

Leu Asp Ser Met Lys Glu Ile Gly Ser Asn Cys Leu Asn Asn Glu Phe
        50              55              60

Asn Phe Phe Lys Arg His Ile Cys Asp Ala Asn Lys Glu Gly Met Phe
65              70              75              80

Leu Phe Arg Ala Ala Arg Lys Leu Arg Gln Phe Leu Lys Met Asn Ser
```

4

Thr Gly Asp Phe Asp Leu His Leu Leu Lys Val Ser Glu Gly Thr Thr
        100   105   .   110

Ile Leu Leu Asn Cys Thr Gly Gln Val Lys Gly Arg Lys Pro Ala Ala
        115   120   125

Leu Gly Glu Ala Gln Pro Thr Lys Ser Leu Glu Glu Asn Lys Ser Leu
    130   135   140

Lys Glu Gln Lys Lys Leu Asn Asp Leu Cys Phe Leu Lys Arg Leu Leu
145   150   155   160

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<211> 48
<212> DNA
<213> Artificial Sequence

<400> 8
gcaagcttgc caccatgttc catgtttctt ttaggtatat ctttggac   48

<210> 9
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ptac1 primer

·<400> 9
atcgagatct aattctcatg tttgacagct tatcat   36

<210> 10
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ptac2 primer

<400> 10
atcgtctaga gctgtttcct gtgtgaaatt gttatccg   38

<210> 11
<211> 20
<212> DNA
<213> Artificial Sequence

6

<220>
<223> Description of Artificial Sequence: tac promoter
      primer

<400> 11
ttcgtgtcgc tcaaggcgca   20

<210> 12
<211> 531
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PS-sIL-7 cDNA

<220>
<221> CDS
<222> (1)..(531)

<400> 12
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atg ttc cat gtt tct ttt agg tat atc ttt gga ctt cct ccc ctg atc   48
Met Phe His Val Ser Phe Arg Tyr Ile Phe Gly Leu Pro Pro Leu Ile
 1               5                  10                  15

ctt gtt ctg ttg cca gta gca tca tct gat tgt gat att gaa ggt aaa   96
Leu Val Leu Leu Pro Val Ala Ser Ser Asp Cys Asp Ile Glu Gly Lys
            20                  25                  30

gat ggc aaa caa tac gag tcc gtt ctg atg gtc agc atc gat caa tta  144
Asp Gly Lys Gln Tyr Glu Ser Val Leu Met Val Ser Ile Asp Gln Leu
        35                  40                  45

ttg gac agc atg aaa gaa att ggt agc aat tgc ctg aat aat gaa ttt  192
Leu Asp Ser Met Lys Glu Ile Gly Ser Asn Cys Leu Asn Asn Glu Phe
    50                  55                  60

aac ttt ttt aaa aga cat cta tgt gat gat aat aag gaa ggt atg ttt  240
Asn Phe Phe Lys Arg His Leu Cys Asp Asp Asn Lys Glu Gly Met Phe
 65                  70                  75                  80

tta ttc cgt gct gct cgc aag ttg agg caa ttt ctt aaa atg aat agc  288
Leu Phe Arg Ala Ala Arg Lys Leu Arg Gln Phe Leu Lys Met Asn Ser
                85                  90                  95

act ggt gat ttt gat ctc cac tta tta aaa gtt tca gaa ggc aca aca  336
Thr Gly Asp Phe Asp Leu His Leu Leu Lys Val Ser Glu Gly Thr Thr
```

7

```
ata ctg ttg aac tgc acc ggc aag gtt aaa gga aga aaa cca gct gcc    384
Ile Leu Leu Asn Cys Thr Gly Lys Val Lys Gly Arg Lys Pro Ala Ala
         115                 120                 125

ctg ggt gaa ccc caa cca aca aag agt ttg gaa gaa aat aaa tct tta    432
Leu Gly Glu Pro Gln Pro Thr Lys Ser Leu Glu Glu Asn Lys Ser Leu
         130                 135                 140

aag gaa cag aaa aaa ctg aat gac tca tgt ttc cta aag aga cta cta    480
Lys Glu Gln Lys Lys Leu Asn Asp Ser Cys Phe Leu Lys Arg Leu Leu
145                 150                 155                 160

caa aag ata aaa act tgt tgg aat aaa att ttg atg ggc act aaa gaa    528
Gln Lys Ile Lys Thr Cys Trp Asn Lys Ile Leu Met Gly Thr Lys Glu
                165                 170                 175

                                                                   531
cac
His
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<210> 13
<211> 177
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: PS-sIL-7 cDNA

<400> 13
Met Phe His Val Ser Phe Arg Tyr Ile Phe Gly Leu Pro Pro Leu Ile
1               5                   10                  15

Leu Val Leu Leu Pro Val Ala Ser Ser Asp Cys Asp Ile Glu Gly Lys
            20                  25                  30

Asp Gly Lys Gln Tyr Glu Ser Val Leu Met Val Ser Ile Asp Gln Leu
         35                  40                  45

Leu Asp Ser Met Lys Glu Ile Gly Ser Asn Cys Leu Asn Asn Glu Phe
      50                  55                  60

Asn Phe Phe Lys Arg His Leu Cys Asp Asp Asn Lys Glu Gly Met Phe
65                  70                  75                  80

Leu Phe Arg Ala Ala Arg Lys Leu Arg Gln Phe Leu Lys Met Asn Ser
                85                  90                  95

Thr Gly Asp Phe Asp Leu His Leu Leu Lys Val Ser Glu Gly Thr Thr
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8

●   ●
            100   105   110

Ile Leu Leu Asn Cys Thr Gly Lys Val Lys Gly Arg Lys Pro Ala Ala
        115   120   125

Leu Gly Glu Pro Gln Pro Thr Lys Ser Leu Glu Glu Asn Lys Ser Leu
        130   135   140

Lys Glu Gln Lys Lys Leu Asn Asp Ser Cys Phe Leu Lys Arg Leu Leu
145   150   155   160

Gln Lys Ile Lys Thr Cys Trp Asn Lys Ile Leu Met Gly Thr Lys Glu
                165   170   175

His

                                 9